66-

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

Applicant's representative would like to thank Examiner Ulrike for her time and very helpful comments during the interview of October 24, 2003.

Rejection under 35 U.S.C. §103

The Examiner maintains the rejection of the claims as being obvious over Kondo et al., combined with Harada et al. and Shirakawa et al. In maintaining the rejection, the Examiner raises the following points each of which will be addressed in turn.

1) The Examiner has acknowledged Applicant's reliance in the May 9, 2003 response on the teaching of Graham et al. that Fas-mediated apoptosis is an unlikely mechanism for cell death in primary biliary cirrhosis. However, the Examiner further notes that the conclusion by Graham et al., "is opinion by the author and is based solely on the low level of expression of CD95/Fas on these cells." Thus, in the Office Action, the Examiner appears to take the position that the conclusion by Graham et al., stating that Fas-mediated apoptosis in an unlikely mechanism, is unsupported by any appropriate evidence in the article. In addition, the Examiner further notes on page 2, spanning page 3, of the Office Action that, "arguments of counsel cannot take the place of evidence of record." The Examiner notes in the Office Action that Graham et

al. teach that they have shown the presence of proteins involved in apoptosis on primary biliary epithelial cells.

As discussed during the interview, the field of the invention was highly unpredictable and controversial at the time of the invention regarding the expression of Fas on liver cells and the association of Fas-mediated apoptosis with primary biliary cirrhosis. The Examiner finds the results and discussions in Graham et al. insufficient to support the position of unpredicatability in the art. However, this unpredictability is not demonstrated solely by the report in Graham et al.

As discussed during the interview, the expression of Fas has been confirmed on a variety of normal organs. Thus, the presence of Fas, per se, cannot be used as a general indicator of pathology. The fact that Fas is also found on normal tissues suggests that Fas-mediated apoptosis plays a role in the normal regeneration and growth of tissue.

As further discussed during the interview, one of the organs where Fas expression has been reported is the biliary epithelial cells of normal liver. Thus, the mere report of the presence of Fas on biliary epithelial cells is not indicative of an involvement of Fas in primary biliary cirrhosis (PBC). One skilled in the art would only conclude that Fas is involved in PBC if the Fas expression was altered on pathological tissue versus normal tissue, with a higher level on the pathological tissue. However, as

evidenced by the following journal articles, which were also discussed during the interview, there have been mutually contradictory reports regarding the expression of Fas on normal versus pathological tissues with PBC.

a) *Leithäuser et al. Laboratory Investigation 69:415-429 (1993)* (copy attached) - Tables 1 and 2 of Leithäuser et al. show that Fas is expressed in a variety of normal human tissue, including biliary epithelial cells of the liver. The staining results of a heptic section are presented in Figure 3D.

b) *Harada et al. Hepatology 26:1399-1405 (1997)* - Page 1403 of Harada et al. teaches that CD95 was not found on normal livers and that "highly up-regulated CD95 expression was...revealed on the injured bile ducts in PBC compared with control livers." Thus, Harada et al. report an increase in Fas expression with PBC.

c) *Graham et al. European J. Gastroenterology & Hepatology 10:553-537(1998)* - As discussed previously, Graham et al. describe on page 555 regarding normal liver samples, "eight of fifteen cases showed moderate staining of biliary epithelium with the anti-Fas/CD95 antibodies" and that "no change in Fas/CD95 staining was noted" in PBC samples. Thus, Graham et al. report no increase in Fas expression with PBC.

d) *Hiramatsu et al. Hepatology 19:1354-1359 (1994)* (copy attached) - Hiramatsu et al. indicate that Fas is not expressed in

either normal or PBC liver tissue. The "Results" section of Hiramatsu et al. state on page 1355 that "no positive stainings were found in 10 samples of normal liver tissues and two samples of tissues of PBC." Thus, Hiramatsu et al. report no Fas expression with PBC (or normal tissue).

Thus, at the time of the invention the connection between Fas and PBC was so controversial that equally reputable scientists reported the contradictory findings that i) Fas is present in normal tissue, ii) Fas is up-regulated in PBC, iii) Fas is not up-regulated in PBC and iv) Fas is not expressed at all on either normal or pathological liver tissue.

Harada et al. concluded that Fas-mediated apoptosis may be involved in the pathology of PBC from the findings that i) Fas expression is higher on biliary epithelial cells in PBC compared to normal tissue, ii) mononuclear cells having a high level of FasL expression may be observed in pathology samples in a greater amount, and iii) the TUNEL index was found to be higher in PBC liver. However, as shown above, the asserted finding of point i) an increased expression of Fas in PBC, is highly controversial and other reports found no change in expression or no expression at all of Fas in PBC livers.

The Examiner suggests on page 2, final three lines of the Office Action that Applicants' arguments are based solely on a low level of Fas expression. However, the conclusion by Harada et al.

that Fas-mediated apoptosis may be involved with the pathology of PBC was also based on their asserted findings regarding the expression of Fas or FasL. Harada et al. considered two criteria, the expression of Fas or FasL and the TUNEL index in PBC. However, these two criteria were considered independently of each other and no direct relationship was tested or demonstrated between Fas-mediated apoptosis and PBC.

Again the experimental results reported by Harada et al. were highly controversial. As discussed above, the first criteria relied on by Harada et al. of increased Fas expression was shown by others to controversial and in dispute. For example, Graham et al. reported no increase in Fas expression and Hiramatsu et al. reported no expression of Fas at all in PBC tissue.

The second criteria relied on by Harada et al. of increased TUNEL index was also controversial and other scientific articles reported that Fas expression does not correspond to the TUNEL activity in bile ducts with some hepatic diseases, including cirrhosis, or, alternatively, that the expression of Fas and TUNEL activity are both negative in bile ducts with hepatic cirrhosis. Attached hereto is an article by Takiya et al., *J. Clin. Pathol.* 48:1093-1097 (1995), wherein the authors report that "histochemistry for Fas antigen and TUNEL tests conducted at the same time showed negative results" for the TUNEL activity in active cirrhosis of the liver. See page 1095, middle of left column and

the table of page 1095 right column of Takiya et al. Takiya et al. further report on page 1097, left column, second paragraph, that "Expression of Fas was not observed where TUNEL was positive. In addition, since Fas antigen was not detected in TGF-β positive sites around the area of atypical bile duct proliferation where apoptosis was found induction of apoptosis might not be mediated by the Fas antigen."

Thus, the second criteria relied on by Harada et al. of TUNEL index was equally controversial in the field with other scientists reporting the exact opposite findings. Given the controversy surrounding both results that were relied upon by Harada et al. to formulate their hypothesis of an involvement of Fas-mediated apoptosis in PBC, and given that Harada et al. did not test a direct relationship between Fas-mediated apoptosis and PBC, one skilled in the art could not predict whether administration of a Fas antagonist would have any efficacy in treating PBC or have any reasonable expectation of success.

Regarding the assertion by the Examiner that "arguments of counsel alone cannot take the place of evidence in the record" Applicants note that the response of May 9, 2003 was not merely arguments of counsel, but submitted Graham et al. as supporting evidence. Submitted herewith are additional journal articles which provide support and evidence all arguments presented and of the unpredicatability of the field of the invention.

2) Kondo et al. is again asserted to teach the involvement of Fas-mediated apoptosis in hepatic cirrhosis. Harada et al. is again asserted to teach the involvement of Fas-mediated apoptosis in primary biliary cirrhosis, which is known to cause bile duct disappearance, the second indication of claim 8. Shirikawa et al. is generally relied on for teaching the therapeutic administration of anti-Fas ligand antibodies.

As discussed above, the possible involvement of Fas-mediated apoptosis was highly controversial with completely opposing positions at the time of the invention. As such, one skilled in the art would have no expectation of success from the teachings in Kondo et al. and Harada et al. when taken in consideration with the general controversy in the field. Shirakawa et al. is further discussed in point 3), below.

3) During the interview of October 24, 2003, Applicants asserted that given the controversy in the field regarding the role of Fas in PBC, one skilled in the art would have no motivation to achieve the invention based on the references or have reasonable expectation of success from the reference teachings. The Examiner clarified her position during the interview and indicated that she felt motivation for achieving the invention could be found in the disclosure in Shirakawa et al. '507 column 42, lines 30-36, of "treating liver dysfunction" by suppressing apoptosis.

Shirakawa et al. disclose in column 42, lines 30-35 that "the dysfunction of the liver tissue caused by the apoptosis of the hepatocytes." (emphasis added) Thus, Shirakawa et al. discusses on only hepatic cells. However, PBC results in the disappearance of the bile ducts. It has been shown that inhibiting the apoptosis of hepatocytes does not lead to the amelioration of bile duct disappearance. Thus, one skilled in the art would have no motivation from the disclosure in Shirakawa et al. regarding hepatocyte apoptosis to use a Fas antagonist to treat or prevent PBC.

The diseases discussed in Shirakawa et al. which pertain to the "the dysfunction in the liver tissue caused by the apoptosis of the hepatocytes" may be typified by hepatitis. In this regard, Kondo et al. also teach in the first sentence of the discussion section found on page 411, left column "in this report, we showed that FasL...is responsible for inducing apoptosis in hepatocytes expressing HBV surface antigen, thus triggering hepatitis." Thus, both Shirakawa et al. and Kondo et al. may suggest or motivate the use of Fas antagonist for treating hepatitis, however neither reference discloses a relationship between Fas antagonists and hepatic cirrhosis.

The pathology of hepatitis is very different from that of hepatic cirrhosis. See page 3, lines 13-24 of the specification, where the differences between hepatitis and hepatic cirrhosis are

discussed. It was unknown at the time of the invention whether or how Fas-mediated apoptosis was involved with the pathology of hepatic cirrhosis. It was further unknown and could not be predicted from what was known in the art, whether the administration of a Fas antagonist would be useful in the prevention and/or treatment of hepatic cirrhosis. For example, as discussed above, Takiya et al. concluded that Fas-mediated apoptosis was not involved with hepatic cirrhosis.

Thus, the disclosure in Shirakawa et al. regarding apoptosis and hepatocytes is not predictive of a role of Fas in bile duct disappearance and hepatic cirrhosis. Nor does Shirakawa et al. provide any motivation for treating hepatic cirrhosis or bile duct disappearance with a Fas antagonist.

One skilled in the art would therefore have no expectation of success or be motivated to achieve the invention from the teachings of the prior art. The present invention is therefore not obvious over the cited references and withdrawal of the rejection is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, PhD. (Reg. No. 40,069) at the telephone number below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

Applicants request a one (1) month extension of time for filing the present response. The required fee is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Gerald M. Murphy, Jr., #28,977

MaryAnne Armstrong, PhD., #40,069

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

GMM/MAA/csm
1110-0279P

Attachment: *Leithäuser et al. Laboratory Investigation 69:415-429 (1993)*
Hiramatsu et al. Hepatology 19:1354-1359 (1994)
Takiya et al. J. Clin. Pathol. 48:1093-1097 (1995)

J Clin Pathol 1995;48:1093–1097

Role of transforming growth factor β1 on hepatic regeneration and apoptosis in liver diseases

S Takiya, T Tagaya, K Takahashi, H Kawashima, M Kamiya, Y Fukuzawa, S Kobayashi, A Fukatsu, K Katoh, S Kakumu

Abstract

***Aims*—To investigate the effects of transforming growth factor β1 (TGF-β1) on regeneration and induction of apoptosis of liver cell and bile duct in various liver diseases.**

***Methods*—Formalin fixed paraffin wax sections of 18 liver tissue samples were obtained by needle biopsy, surgery, or necropsy; these included six liver cirrhosis, three obstructive jaundice; five fulminant hepatitis, one subacute hepatitis, and three normal liver. Expression of TGF-β1, apoptosis related Le^y antigen, Fas antigen, a receptor for tumour necrosis factor, and biotin nick end labelling with terminal deoxynucleotidyl transferase mediated dUTP (TUNEL) for locating DNA fragmentation, was investigated histochemically.**

***Results*—TGF-β1 was expressed in areas of atypical bile duct proliferation, where bile duct continuously proliferated from liver cells. In occlusive jaundice and fulminant hepatitis, TUNEL was positive in nuclei and cytoplasm of metaplastic cells which formed incomplete bile ducts, and these cells appeared to extend from TGF-β1 expressing liver cells. Fas antigen was found only on the cell membrane of proliferated bile duct in fulminant hepatitis, which differed from TGF-β1 and TUNEL positive areas. Le^y antigen was expressed in liver cell and bile duct at the areas with atypical bile duct proliferation, but its coexpression with TUNEL was rare.**

***Conclusions*—TGF-β1 plays a role in the arrest of liver cell regeneration and atypical bile duct proliferation, and in areas of rapidly progressing atypical bile duct proliferation, such as in fulminant hepatitis or bile retention. Apoptosis appears to be induced by TGF-β1. This phenomenon may account for the inadequate hepatic regeneration that occurs with liver disease.**

(*J Clin Pathol* 1995;48:1093–1097)

Keywords: TGF-β1, apoptosis, hepatic regeneration.

1st Department of Internal Medicine, Aichi Medical University, Aichi Prefecture, Japan
S Takiya
T Tagaya
K Takahashi
H Kawashima
M Kamiya
Y Fukuzawa
S Kobayashi
A Fukatsu
K Katoh

3rd Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan
S Kakumu

Correspondence to:
Dr Satoshi Takiya,
First Department of Internal Medicine, Aichi Medical University, Nagakute-cho, Aichi-gun, Aichi Prefecture 480-11, Japan.

Accepted for publication
10 October 1994

Transforming growth factor β1 (TGF-β1), a peptide widely distributed in tissues, is known to have diverse biological activities. TGF-β1 may regulate the proliferation of epithelial and mesenchymal cells as well as immunocompetent cells. TGF-β1 also exerts stimulatory effects on the deposition of extracellular matrix and the proliferation of fibroblasts; these effects are typically shown in wounded tissue repair.[1]

One of the important biological activities of TGF-β1 is inhibition of liver cell regeneration.[2–8] However, it also promotes the proliferation of fibroblasts, which seems to cause fibrosis of the liver.[9–12] Therefore recent research on the role of TGF-β1 in liver diseases has been concentrated chiefly on the progression of fibrosis in this disease,[13–15] although fibrosis is not a simple phenomenon—it should be recognised as a series of events, including fibril production and liver cell regeneration.

Recently, TGF-β1 has been shown to induce liver cell apoptosis through the inhibition of DNA synthesis by liver cells.[16–21] Apoptosis—programmed cell death—plays a key role in developmental biology and in the maintenance of the steady state in continuously renewing tissue. Cell condensation and fragmentation into a number of membrane bound "apoptotic bodies", initially containing well preserved organelles and often condensed chromatin, are hallmarks of apoptotic cell death when identified by electron microscopy. So far, the pathological significance of this phenomenon in liver disease remains unclear. In order to clarify the role of TGF-β1 on regeneration and apoptosis of liver cells and bile duct, we conducted an immunohistochemical analysis of the expression of TGF-β1, Le^y antigen,[22 23] an apoptosis related marker, and Fas antigen,[24–26] one of the tumour necrosis factor (TNF) receptors, in various types of liver disease. The terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling method (TUNEL),[27–29] which recognises DNA fragmentation, was also used to analyse the site of apoptosis.

Methods

The study was conducted on 18 liver tissue samples obtained by needle biopsy, surgery, or necropsy. These consisted of five cases of fulminant hepatitis (type B), one of subacute hepatitis (type B), six of active cirrhosis of the liver (type C), three of obstructive jaundice (cancer of the pancreatic head), and three of normal liver (normal part of the surgically resected liver with benign tumour). Type B and type C hepatitis were serologically diagnosed by the presence of hepatitis B surface antigen and hepatitis C virus antibody (assessed with a

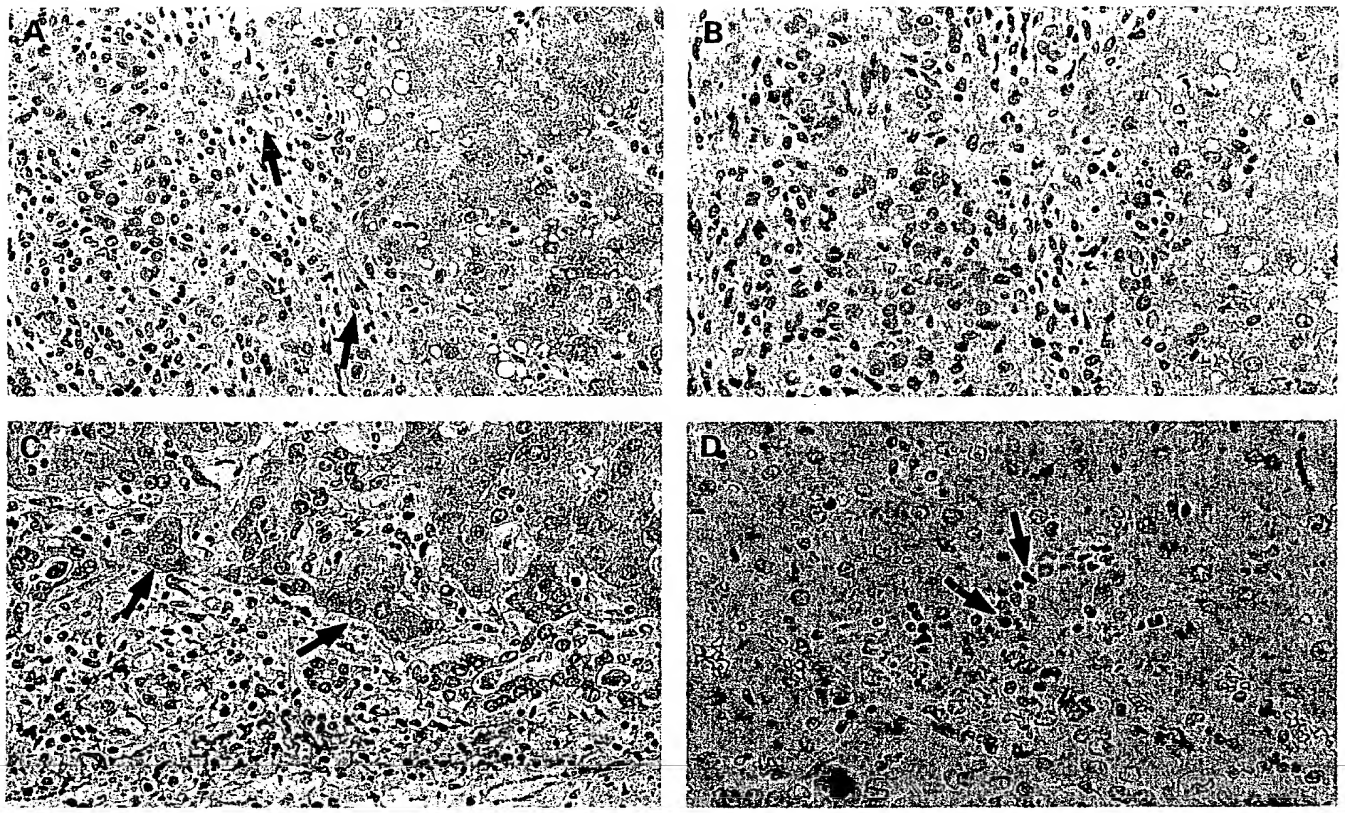

Figure 1 TGF-β1 was expressed in the cytoplasm of liver cells and the metaplastic bile ducts in regenerative nodules of active cirrhosis of the liver (indicated by arrows) (A), AEC (× 100). Le^y antigen also gave positive findings in some liver cells and proliferated bile ducts (B), AEC (× 100). Morphologically incomplete bile duct continuous with TGF-β positive liver cells was observed in obstructive jaundice (indicated by arrows) (C), AEC (× 100). Bile duct nuclei were positive for TUNEL (shown by arrows) (D), AEC (× 100).

second generation assay, Abbott Laboratories), respectively.

The tissue was fixed for 24 hours with 10% buffered formalin solution, embedded in paraffin wax, and thin sections measuring 2–3 μm in thickness were prepared. Deparaffinisation was carried out with xylene, and the sections were then hydrated using an alcohol series. They were thoroughly rinsed with purified water. Immunohistochemical staining was conducted by the following method. Intrinsic peroxidase was inactivated for 10 minutes with 0·3% H_2O_2, and rinsed with phosphate buffered saline (PBS, 1/15 mol/l, pH 7·2). The sections were incubated with properly diluted rabbit or mouse serum for 30 minutes at room temperature to avoid non-specific binding of second antibodies. Sections were reacted overnight at 4°C with anti-human TGF-β1 rabbit polyclonal antibody (4 μg/ml, King Brewing), antihuman Le^y mouse monoclonal antibody (20 μg/ml, BM-1, Japan Immunoresearch Laboratory Co),[29] or anti-human Fas mouse monoclonal antibody (20 μg/ml, CH-11, Medical and Biological Laboratory Co) followed by a reaction for 30 minutes at 20°C using a biotinated second antibody kit (IMMUNON immunostaining systems, Shandon Lipshaw Inc). The reaction product was visualised using a mixed solution of sodium acetate buffer including 0–3% H_2O_2 and 3-amino-9-ethylcarbazole (AEC). Sections were further stained with haematoxylin and observed with Olympus BH-2 microscope.

TUNEL was carried out according to the method of Gavrieli *et al.*[27 29] After fixing with 10% formalin buffer, sections were deparaffinated and hydrated. Sections were reacted with proteinase K (20 μg/ml) at room temparature for 20 minutes in 100 mM trisHCl buffer (pH 7·4), and rinsed in purified water including 3% H_2O_2 in order to inactivate intrinsic peroxidase activity. After rinsing with terminal deoxynucleotidyl transferase (TdT) buffer (potassium cacodylate 100 mM, $CoCl_2$ 2 mM, dithiothreitol 0·2 mM, pH 7·2), sections were incubated for 60 minutes at 37°C with TdT solution (TdT 0·3 equivalent U/μl biotinylated uridine triphosphate 0·04 nmol/μl). They were rinsed in buffer solution (sodium citrate 30 mM, NaCl 300 mM) for 15 minutes and then in purified water and PBS. Non-specific reaction was blocked with 10% normal rabbit serum. Sections were then reacted with streptoavidin labelled with peroxidase for 30 minutes at room temperature. Positive reaction was visualised using AEC mixed solution, and the nuclei were stained with haematoxylin. As a positive control, a reaction with DNase 1 (0·7 μg/ml potassium cacodylate buffer, pH 7·2) was conducted before the addition of TdT reaction solution, and as a negative control, TUNEL was conducted using a solution without TdT.

For the observation with a confocal laser scanning microscope (LSM 410, Carl Zeiss), fluorescent (FITC) labelled streptoavidin was used instead of peroxidase conjugate.

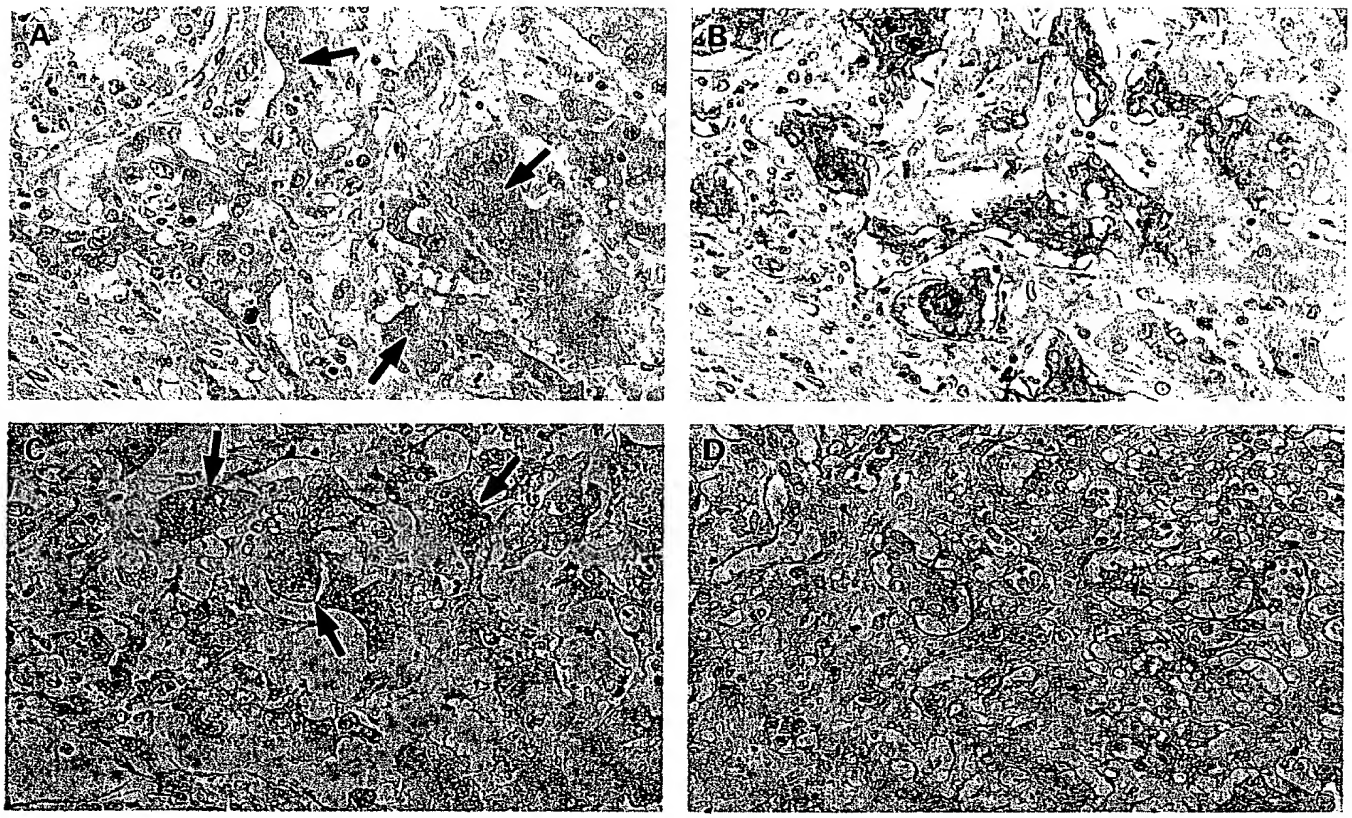

Figure 2 Bile duct proliferation extending from TGF-β1 expressing liver cells showed incomplete morphology in fulminant hepatitis. Some of the nuclei were destroyed (indicated by arrows) (A), AEC (× 100). Le^y antigen was seen in the same area (B) (× 100). Cytoplasm of liver cell was positive for TUNEL (arrows) (C) (× 100). Fas antigen showed positive in the cell membrane of bile ducts which may proliferate at the other sites described above (D), AEC (× 100).

Results

In the normal part of the liver, TGF-β1, Fas antigen, and TUNEL were negative in all cases. Le^y antigen was also negative in all cases with the exception of inflammatory cells. In the area of regenerative nodules in active cirrhosis of the liver, TGF-β1 was observed not only in the cytoplasm of liver cells, but also of the metaplastic bile duct epithelium (fig 1A), where proliferative changes of liver cells to bile duct epithelial cells were noted. Le^y antigen was also expressed in the cytoplasm of some liver cells and in metaplastic bile duct epithelium, although the bile duct structure was recovered (fig 1B). Histochemistry for Fas antigen and TUNEL tests conducted at the same time showed negative results.

In cases with obstructive jaundice, TUNEL was positive in the nuclei of metaplastic cells, which formed incomplete bile ducts (fig 1D) and these cells appeared to extend from TGF-β1 expressing liver cells (fig 1C). Le^y antigen was positive in the cytoplasm of metaplastic bile duct forming cells, but Fas antigen was negative.

In cases of fulminant hepatitis, TGF-β1 was expressed both in liver cells and in adjacent bile duct forming cells, which were highly metaplastic (fig 2A). Some nuclei of cells in these areas showed morphological changes with an expression of Le^y antigen (fig 2B). TUNEL was positive in the cytoplasm of these cells (fig 2C).[28] Fas antigen was, however, positive only on the cell membrane of bile duct forming cells at the sites of inflammatory cell phagocytosed bilirubin (fig 2D). Proliferated bile duct forming cells were negative for TUNEL, but positive for Le^y antigen in the cytoplasm.

Confocal laser scanning microscopy revealed various stages of apoptosis. In an early stage, morphology of nucleolus and nucleoli was relatively preserved, and the FITC positive sites showed the finding of DNA fragmentation (fig 3A). As apoptosis progressed, the following findings were observed: outflow of the nuclear contents (fig 3B), migration of the TUNEL positive substance from the nucleus to the cytoplasm (fig 3C), and disappearance of the nucleus. TUNEL was positive only in the

Summary of expession of TGF-β 1, Le^y Ag, Fas Ag, and TUNEL in liver cell and bile duct in various conditions of liver disease

	TGF-β1	*Le^{y}Ag*	*Fas Ag*	*TUNEL*
Active cirrhosis liver cell—bile duct (atypical)	+	+	−	−
Obstructive jaundice liver cell—incompletely formed bile duct (atypical)	+	+	−	+
Fulminant hepatitis liver cell—incompletely formed bile duct (atypical)	+	+	−	+
Proliferated bile duct	± ~ −	+	+	−

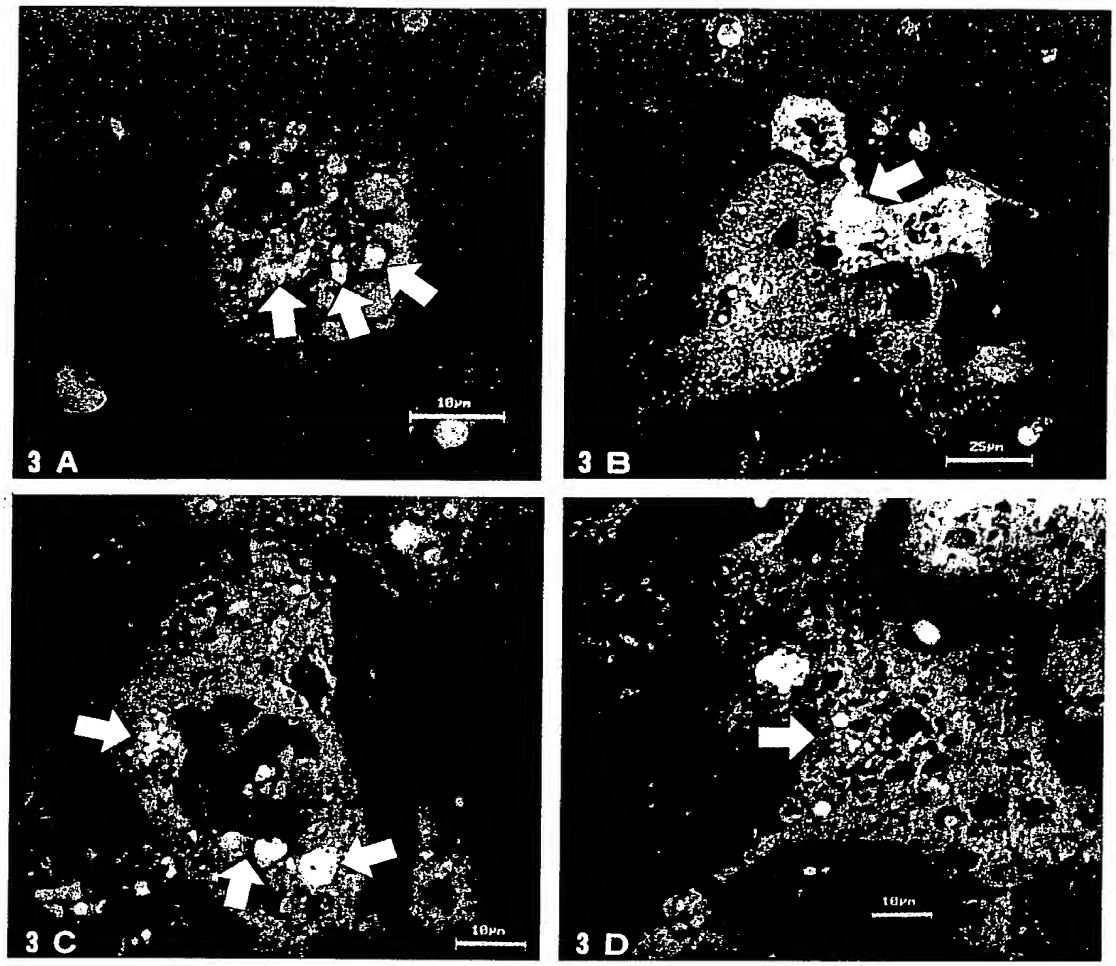

Figure 3 Areas shown in panels (A), (B), and (C) are the same as in the corresponding panels in fig 2. Fragmentation of DNA was noted in fulminant hepatitis under confocal laser scanning microscopy with TUNEL (shown by arrows) (A). Outflow of nuclear contents is indicated with arrows (B). Migration of TUNEL positive substances to cytoplasm is shown by arrows (C). TUNEL positive findings in the cytoplasm of cells with destroyed nuclei are indicated with arrows in (D).

cytoplasm of liver cells (fig 3D), which migrated into the area with incomplete formation of bile ducts.

Discussion

In the lesions with marked bile duct proliferation, which was typically seen in the regenerative nodules of active cirrhosis of the liver, obstructive jaundice, and fulminant hepatitis, two possible origins of the cells forming bile ducts have been described, namely atypical ductular proliferation, in which liver cells transform to the bile duct epithelium, and typical ductular proliferation, in which the remaining bile duct cells proliferate and form bile ducts.[30–32] The fact that liver cells transform into the bile duct forming cells in an early stage is confirmed experimentally. Tomoyori *et al* reported that in the hepatectomised rat, liver cells showed marked expansion of the bile canaliculus and formed an acinar arrangement.[33] A similar transformation has also been described in experimental transplantation of the liver into the spleen in rats.[34]

Our study showed that TGF-β1 was expressed on bile duct forming cells and liver cells in areas of atypical ductular proliferation (table). This suggests that TGF-β1 is an important factor modulating the regeneration mechanism of the bile duct system as well as the parenchymal cells of the liver. However, the sites of TGF-β1 detection may not be the sites of synthesis. For example, the cells other than those detected immunohistochemically (for example, the endothelial cell) may be the true sites of synthesis; this can be proved by techniques such as in situ hybridisation.

Atypical ductular proliferation was observed when the number of remaining small bile duct cells was insufficient to proliferate in situations that result in a decrease in the absolute number of liver cells, destruction of liver architecture, and marked bile retention. With an increase in bile retention, more rapid proliferation of bile duct cells is required.[35] When pronounced destruction of the liver architecture takes place, such as in fulminant hepatitis, regeneration of liver cell should in theory be coordinated with that of bile duct system. However, the latter appeared strikingly enhanced in our study. It is conceivable that the large amounts of TGF-β1 noted are necessary to inhibit excessive bile duct proliferation.

In the present study, we could detect the morphological occurrence of apoptosis by laser scanning microscopy at the sites where metaplastic bile duct cells had contact with liver cells. Le^y antigen was expressed in the cytoplasm of liver cells in active cirrhosis of the liver together with TGF-β1, and it was also expressed in metaplastic cells seen in atypical bile duct proliferation. However, concomitant positive findings of Le^y antigen and TUNEL were only observed in metaplastic cells with incomplete bile duct formation which had contact with TGF-β1 positive liver cells in obstructive jaundice and fulminant hepatitis. On the basis of these findings, we conclude that the detection of Le^y antigen is a useful method of recognising the possible induction of apoptosis, but it was difficult to confirm the process by this expression alone, as display of the Le^y antigen does not always represent chromatin condensation and DNA fragmentation of the nuclei.

Expression of Fas antigen was not observed at the sites where TUNEL was positive. In addition, since Fas antigen was not detected at TGF-β1 positive sites around the area of atypical bile duct proliferation where apoptosis was found, induction of apoptosis might not be mediated by the Fas antigen. It has been reported that the bile duct, which is formed by cell proliferation and differentiation in experimental obstructive jaundice, disappears due to the induction of apoptosis after the obstruction is relieved.[36 37] In the present study, TGF-β1 was not detected in the mature, differentiated metaplastic bile duct, and in the stage when apoptosis was detected. This finding is at variance with published reports.

From these results, we suggest that TGF-β1 plays a role in the arrest of liver cell regeneration and in atypical bile duct proliferation. In severe necrosis of liver tissue with marked destruction of liver architecture, seen typically in fulminant hepatitis or bile retention, apoptosis was observed at the site of rapid atypical bile duct proliferation. This could be the result of the inhibition of DNA synthesis by TGF-β1 and this phenomenon may account for the regenerative phase of liver disease.

1 Massaque J. Transforming growth factor-β family. *Annu Rev Cell Biol* 1990;6:597–641.
2 Tomita Y, Nakamura T, Ichihara A. Control of DNA synthesis and ornithine decarboxylase activity by hormones and amino acids in primary cultures of adult rat hepatocytes. *Exp Cell Res* 1981;125:363–71.
3 Nakamura T, Tomita Y, Ichihara A. Density-dependent growth control of adult rat hepatocytes in primary culture. *J Biochem* 1983;94:1029–35.
4 Nakamura T, Ichihara A. Control of growth and expression of differentiated functions of mature hepatocytes in primary culture. *Cell Struct Func* 1985;10:1–16.
5 Nakamura T, Arakaki R, Ichihara A. Interleukin-1β is a potent growth inhibitor of adult rat hepatocytes in primary culture. *Exp Cell Res* 1988;179:488–97.
6 Braun L, Mead J, Panzica M, Mikumo R, Bell GI, Fausto N. Transforming growth factor β mRNA increases during liver regeneration. A possible paracrine mechanism of growth regulation. *Proc Natl Acad Sci USA* 1988;85:1539–43.
7 Russell WE, Coffey RJ, Ouellette AJ, Moses HL. Type 1 β transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat. *Proc Natl Acad Sci USA* 1988;85:5126–30.
8 Zarnegar R, Michalopoulos G. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. *Cancer Res* 1989;49:3314–20.
9 Czaja MJ, Weiner FR, Flanders KC, Giambrone MA, Wind R, Biempica L, *et al.* In vitro and in vivo association of transforming growth factor-β1 with hepatic fibrosis. *J Cell Biol* 1989;108:2477–82.
10 Weiner FR, Giambrone M, Czaja MJ, Shah A, Annoni G, Takahashi S, *et al.* Ito-cell gene expression and collagen regulation. *Hepatology* 1990;11:111–17.
11 Nakatsukasa H, Nagy P, Evarts RP, Hsia C, Marsden E, Thorgeirsson SS. Cellular distribution of transforming growth factor-β1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *J Clin Invest* 1990;85:1833–43.
12 Maher JJ, McGuire RF. Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. *J Clin Invest* 1990;86:1641–8.
13 Nagy P, Schaff Z, Lapis K. Immunohistochemical detection of transforming growth factor-β1 in fibrotic liver diseases. *Hepatology* 1991;14:269–73.
14 Castilla A, Prieto J, Fausto N. Transforming growth factors β1 and α in chronic liver disease. Effects of interferon alfa therapy. *N Engl J Med* 1991;324:933–40.
15 Annoni G, Weiner FR, Zern MA. Increased transforming growth factor-β1 gene expression inhuman liver disease. *Hepatology* 1992;14:259–64.
16 Oberhammer F, Bursch W, Parzefall W, Breit P, Erber E, Stadler M, *et al.* Effect of transforming growth factor β on cell death of cultured rat hepatocytes. *Cancer Res* 1991; 51:2478–85.
17 Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, *et al.* Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. *Proc Natl Acad Sci USA* 1992; 89:5408–12.
18 Oberhammer F, Fritsch G, Pavelka M, Froschl G, Tiefenbacher R, Purchio T, *et al.* Induction of apoptosis in cultured hepatocytes and in the regressing liver by transforming growth factor-β1 occurs without activation of an endonuclease. *Toxicol Lett* 1992;64/65:701–4.
19 Bursch W, Oberhammer F, Jirtle RL, Askari M, Sedivy R, Grasl-Kraupp B, *et al.* Transforming growth factor-β1 as a signal for induction of cell death by apoptosis. *Br J Cancer* 1993;67:531–6.
20 Oberhammer F, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio T, *et al.* Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. *J Cell Sci* 1993;104:317–26.
21 Oberhammer F, Bursch W, Tiefenbacher R, Frosche G, Pavelka M, Purchio T, *et al.* Apoptosis is induced by transforming growth factor-β1 within 5 hours in regressing liver without significant fragmentation of the DNA. *Hepatology* 1993;18:1238–46.
22 Abe K, McKibbin JM, Hakomori S. The monoclonal antibody directed to difucosylated type 2 chain (Fucα1→2Gα1β1→4[Fucα1→3]G1lcNAc Y determinant. *J Biol Chem* 1983; 58:11793–7.
23 Adachi M, Hayami M, Kashiwagi N, Mizuta T, Ohta Y, Gill M J, *et al.* Expression of Le^y antigen in human immunodeficiency virus-infected human T cell lines and in peripheral lymphocytes of patients with acquired immune deficiency syndrome (AIDS) and AIDS-related complex (ARC). *J Exp Med* 1988;167:323–31.
24 Yonehara S, Ishii A, Yonehara M. A cell killing monoclonal antibody (anti Fas) to a cell surface antigen co-downregulated with the receptor of tumour necrosis factor. *J Exp Med* 1989;169:1747–56,
25 Itoh N, Nagata S. A novel protein domain required for apoptosis. *J Biol Chem* 1993;268:10932–7.
26 Ogasawara J, Fukunaga RW, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, *et al.* Lethal effect of the anti-Fas antibody in mice. *Nature* 1993;364:806–9.
27 Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493–501.
28 Wijsman JH, Jonker RR, Keijzer R, Velde CJH, Cornelisse CJ, Dierendonck JH. A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. *J Histochem Cytochem* 1993;41:7–12.
29 Hiraishi K, Suzuki K, Hakokmori S, Adachi M. Le^y antigen expression is correlated with apoptosis (programmed cell death). *Glycobiology* 1993;3:381–90.
30 Uchida T, Peters RL. The nature and origin of proliferated bile ductules in alcoholic liver disease. *Am J Clin Pathol* 1983;79:326–33.
31 Nakanuma Y, Ohta G. Immunohistochemical study on bile ductular proliferation in various hepatobiliary disease. *Liver* 1986;6:206–11.
32 Thung SN. The development of proliferating ductular structures in liver disease. *Arch Pathol Lab Med* 1990;114: 404–11.
33 Tomoyori T, Ogawa K, Mori M, Onoe T. Ultrastructural changes in the bile canaliculi and the lateral surfaces of rat hepatocytes during restorative proliferation. *Virchows Arch B Cell Pathol* 1983;42:201–11.
34 Ebata H, Onodera K, Sawa M, Mito M. A study of liver regeneration using fetal rat liver tissue transplanted into the spleen. *Jpn J Surg* 1988;18:540–7.
35 Shibayama Y. Factors producing bile infarction and bile duct proliferation in biliary obstruction. *J Pathol* 1990; 160:57–62.
36 Bhathal PS, Gall JAM. Deletion of hyperplastic biliary epithelial cells by apoptosis following removal of the proliferative stimulus. *Liver* 1985;5:311–25.
37 Gall JAM, Bhathal PS. Origin and involution of hyperplastic bile ductules following total biliary obstruction. *Liver* 1990;10:106–15.

Immunohistochemical Detection of Fas Antigen in Liver Tissue of Patients with Chronic Hepatitis C

NAOKI HIRAMATSU, NORIO HAYASHI, KAZUHIRO KATAYAMA, KIYOSHI MOCHIZUKI, YUKO KAWANISHI, AKINORI KASAHARA, HIDEYUKI FUSAMOTO AND TAKENOBU KAMADA

First Department of Medicine, Osaka University Medical School, Osaka 565, Japan

Apoptosis is a type of cell death that occurs in acute or chronic hepatitis. It has been suggested to be mediated through Fas antigen. To evaluate the role of apoptosis on liver injury of chronic hepatitis C, we studied the expressions of Fas antigen and hepatitis C virus antigen (core antigen) immunohistochemically. Forty liver biopsy samples from patients with type C chronic liver disease were immunostained for Fas antigen and hepatitis C virus antigen. Expression of Fas antigen was found mainly in the cytoplasm of hepatocytes, and these positive cells were found particularly among infiltrating lymphocytes at the advancing edges of "piecemeal necrosis." The histological activity index showed inflammation of both portal and periportal areas to be more severe in the Fas antigen–positive samples than in the Fas antigen–negative ones ($p < 0.05$ and $p < 0.001$, respectively). Furthermore, semiquantitative analysis revealed more expression of Fas antigen in the liver tissues with active inflammation than in those without it ($p < 0.01$). The prevalence of Fas antigen expression in the hepatitis C virus antigen–positive group was higher than that in the hepatitis C virus antigen–negative group ($p < 0.05$). Our findings suggest that Fas antigen expression (apoptosis) plays an important role in inflammation in the hepatitis C virus–infected liver, particularly in the active inflammation of chronic hepatitis C. (HEPATOLOGY 1994;19:1354-1359.)

In 1989, the genome of hepatitis C virus (HCV) was cloned by Choo et al. (1), and specific diagnostic tools for hepatitis C have been developed (i.e., serological assays for HCV-related antibodies [2-4] and an assay of reverse transcription and polymerase chain reaction [RT-PCR] for detecting HCV RNA in sera [5, 6]). HCV genomes have been detected in livers with HCV infection by means of RT-PCR (7, 8) and HCV-infected hepatocytes have been detected in the human liver by means of immunohistochemical methods (9) and *in situ* hybridization (10). Despite such rapid advances in research on hepatitis C, the mechanism of the liver injury it causes remains to be clarified.

Cell death is known to take two forms, *necrosis* and *apoptosis* (11, 12). In 1972, Kerr et al. (13) described different morphologies for programmed and pathological cell death, and Wyllie (14) laid the basis for biochemical investigations of programmed cell death. The morphology of programmed cell death is called apoptosis, which differs morphologically from necrosis on observation under light or electron microscopy. In addition, internucleosomal chromatin cleavage (DNA fragmentation) is observed as a ladder on gel electrophoresis with DNA lengths that are integer multiples of 180 to 200 bp along with morphologic chromatin condensation of apoptosis (14, 15). Apoptosis has been shown to be the type of cell death in a number of diseases, including acute and chronic hepatitis, in which the apoptotic bodies have traditionally been referred to as acidophilic or Councilman bodies (16, 17); PBC (18); and lichen planus (19). In the liver, importance has been attached to apoptosis because this is the form found by light and electron microscopy, especially among the infiltrating lymphocytes at the advancing edges of areas of piecemeal necrosis in CAH (12, 16). However, the role of apoptosis in chronic hepatitis could not be studied adequately because apoptotic bodies are histologically visible for only a few hours (12, 15, 16).

Recently, Itoh et al. (20) cloned cDNA for the human Fas antigen, which is a cell surface protein that mediates apoptosis with treatment of the antibody to Fas antigen (anti-Fas). Molecular cloning has revealed that the Fas antigen belongs to the receptor family that includes tumor necrosis factor (TNF) receptor, nerve growth factor receptor, B cell CD40 antigen and T-cell OX40 antigen. Northern analysis indicates Fas antigen messenger RNA is expressed in a limited number of tissues, including the thymus, liver, heart, lung and ovary of normal mouse (21). Mice carrying the lymphoproliferation mutation, in which lymphoadenopathy and a systematic lupus erythematosus–like autoimmune disease develop, have been shown to have defects in the Fas antigen gene, indicating an important role of Fas antigen in the negative selection (apoptosis) of autoreactive T cells in mouse thymus (22). On the other hand,

Received August 4, 1993; accepted December 31, 1993.

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

Address reprint requests to: Norio Hayashi, M.D., First Department of Medicine, Osaka University Medical School, Yamadaoka 2-2, Suita, Osaka 565, Japan.

0270-9139/94 $3.00 + 0 31/1/54558

anti-Fas was found to kill the chronically human immunodeficiency virus (HIV)–infected cells, which were shown to be strikingly sensitive to the anti-Fas antibody in comparison with uninfected cells (23). This indicates that the Fas antigen is not only a molecule constitutively expressed on a cell programmed for death by apoptosis but also an inducible molecule that can be expressed *de novo* on a cell under abnormal circumstances. This study was conducted to examine the expression of Fas antigen in liver tissues with hepatitis C with the object of evaluating the role of apoptosis in chronic hepatitis C.

MATERIALS AND METHODS

Patients. Liver tissue was obtained under peritoneoscopy from 42 patients and by surgical resection of the liver from 10 patients. Biopsies were performed for diagnostic purposes, and informed consent was obtained from each patient. Surgery was performed for resection of space occupying lesions (metastatic lesion in liver of colon cancer, 5; liver hemangioma, 2; cholangiocellular carcinoma 2; hepatolithiasis, 1) from the normal liver, and these normal liver tissues were used for this study. Forty patients (27 males and 13 females), ranging in age from 15 to 68 yr (mean, 51.0 ± 12.0 yr), were seropositive for the antibody to HCV (anti-HCV) according to results of a second-generation ELISA. None of the anti-HCV-positive patients had any evidence of chronic hepatitis B (all were negative for HBsAg and 1:200-diluted antibody to HBcAg) or autoimmunity. (All were negative for antinuclear antibody, and all serum levels of gammaglobulin were less than 2.5 gm/dl.) The mean level of serum ALT was 128.0 ± 18.4 IU/L. The histology of the liver samples from the anti-HCV–positive patients indicated chronic persistent hepatitis (CPH) in 16 and CAH in 24, including CAH with cirrhosis in 2. Two patients were seropositive for antimitochondrial antibody and were diagnosed as having PBC (stage 1) on histological study. All liver biopsy specimens were examined independently by two hepatologists unaware of the clinical findings. The specimens were also evaluated according to the histology activity index (HAI) scoring system of Knodell et al. (24).

Preparation of Antibodies to Core Antigen of HCV and Fas Antigen. Details of preparation of antibody to core antigen (anti-core) have been reported elsewhere (9). In brief, an HCV cDNA fragment (25) spanning the core region (nucleotides 369 to 700) was inserted into a plasmid expression vector and expressed in *Escherichia coli*. We obtained monoclonal antibody to this recombinant protein by injecting it into mouse. Anti-Fas (IgM fraction of the mouse monoclonal antibody) was purchased from MBL Co. (Nagoya, Japan [26]).

Immunohistochemical Procedures. All samples were fixed with Zamboni solution for 24 hr at 4° C. They were then frozen and cut into 8-μm slices with a cryostat. The sections were incubated with 1:200-diluted anti-Fas or 1:500-diluted anti-core for 24 hr. Next they were incubated with biotinylated anti-mouse IgM for anti-Fas or biotinylated anti-mouse IgG for anti-core; avidin-biotin peroxidase complex was then applied (Vectastain ABC kits; Vector Laboratories, Burlingame, CA), followed by diaminobenzidine-H_2O_2 substrate and counterstaining with hematoxylin. The entire procedure was performed at room temperature. The specificity of anti-core had been confirmed previously (9), and that of anti-Fas was examined elsewhere (26). The serial sections were stained similarly with an inappropriate agent such as another mouse monoclonal antibody (the antibody to 5-bromo-2′-deoxyuridine; Amersham International, Buckinghamshire, UK) or mouse gamma-globulin (Sigma Chemical Co., St. Louis, MO) instead of the mouse monoclonal antibodies used in this study. The immunostaining was also performed without the primary antibodies. Several samples were preincubated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature so that we might inactivate the endogenous peroxidase before performing incubation with the primary antibodies. The degree of Fas antigen expression in the periportal area was classified into four groups: none, positive cells in less than ⅓ of the hepatocytes in the circumference of most portal tracts, involvement of ⅓ to ⅔ of the circumference of most portal tracts, involvement of more than ⅔ of the circumference of most portal tracts. The degree of Fas antigen expression in the intralobular area was classified into three groups: none, the number of positive cells less than ⅓ of the number of all hepatocytes in the lobule and involvement more than ⅓ of the number of all hepatocytes in the lobule.

Statistical Analysis. Values are expressed as mean ± S.E.M. Means were compared with the Mann-Whitney U test and the χ^2 test. All p values are two-sided.

RESULTS

Expression of Fas Antigen. Fas antigen was expressed in the HCV-infected liver. Hepatocytes were strongly stained in the cytoplasm (partly in the membrane) and these positive cells were found particularly among the infiltrating lymphocytes, at the advancing edges of areas of piecemeal necrosis (Fig. 1a). A portion of bile duct cells was also stained in the cytoplasm. No positive reaction was found in the cells after immunostaining with mouse gamma-globulin or another mouse monoclonal antibody (the antibody to 5-bromo-2′-deoxyuridine) instead of specific antibodies or after omission of the primary antibody. After immunostaining with anti-Fas, we found heavily stained cells other than hepatocytes in the lobules, but such stained cells did not appear when the samples were preincubated with hydrogen peroxide in methanol. No positive stainings were found in 10 samples of normal liver tissues and two samples of liver tissues of PBC.

Fas Antigen Expression and Histological Appearance. The prevalence of Fas antigen expression in the liver samples of the patients with type C chronic liver disease is shown in Table 1. Of the 40 samples, 26 were positive on Fas antigen staining (65%). Only six samples were positive in 16 of the patients with CPH (38%); 20 samples were positive in 24 of the patients with CAH (83%). A higher prevalence of Fas antigen expression was found in liver tissue with active inflammation (CAH) than in that without it (CPH) ($p < 0.01$). However, no significant difference in the mean ALT levels was seen between the Fas antigen–positive group (144.1 ± 27.4 IU/L) and the Fas antigen–negative group (102.7 ± 17.9 IU/L).

The distribution of Fas antigen expression was divided into two main classes—that is, expression in the periportal area (Fig. 1a) and in the intralobular area (Fig. 1c). Most of the cases with CAH revealed both periportal expression and intralobular expression; those with CPH showed only intralobular expression.

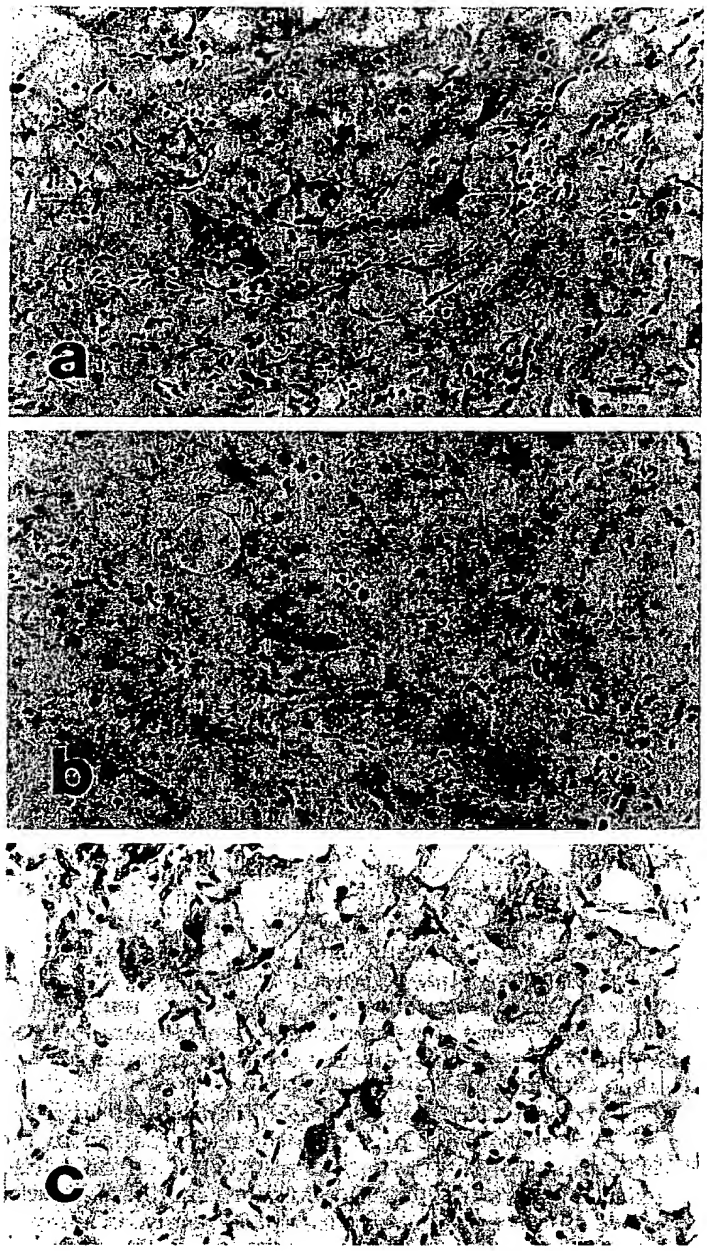

FIG. 1. Tissue sections from a patient with chronic hepatitis C immunostained to reveal cells expressing (a) Fas antigen and (b) HCV antigen (core antigen), which are found among the infiltrating lymphocytes, at the advancing edges of piecemeal necrosis (periportal area). (c) Expression of Fas antigen in the lobule. Parts (a) to (c) show the positive decoration in the cytoplasm of the hepatocytes (indirect immunohistochemical method, counterstained with Mayer's hematoxylin; original magnification ×400).

To clarify whether Fas antigen expression is increased in the inflamed liver tissue, we performed semiquantitative analysis (Table 2). In the periportal area, more Fas antigen expression was indicated in the liver tissues of the CAH patients than in those of the CPH patients ($p < 0.01$), and the liver tissues of the CAH patients also tended to express more Fas protein in the intralobular area, although no significant difference was found. For further study, we conducted separate evaluation of the relationship between the periportal expression of Fas antigen and histological appearance scored according to Knodell's HAI system (24) and the relationship between the intralobular expression and histological appearance in the lobule scored by the same system. The former result is shown in Table 3. Periportal expression of Fas antigen was found in 18 of the 40 samples (45%), and inflammation of both periportal and portal areas were found to be more severe in the samples of the Fas-antigen–positive group than in those of the Fas-antigen–negative group ($p < 0.001$ and $p < 0.05$, respectively), but no significant difference was seen between periportal expression of Fas antigen and lobular inflammation. Intralobular expression of Fas antigen was found in 22 of the 40 samples (55%), but no significant difference of lobular inflammation was observed between the Fas antigen (intralobular expression)–positive group and the Fas antigen–negative group (1.8 ± 0.3 and 1.4 ± 0.2, respectively).

TABLE 1. Clinical and biochemical characteristics of 40 patients with type C chronic liver disease, classed according to Fas antigen expression

Clinical features	Fas antigen–positive (n = 26)	Fas antigen–negative (n = 14)
F/M	7/19	6/8
Age (yr)[a]	53.7 ± 2.2[b]	46.7 ± 3.4
ALT (IU/L)[a]	144.1 ± 27.4[b]	102.7 ± 17.9
Histological findings		
CPH (n = 16)	6 (38%)[c]	10 (62%)
CAH (n = 24)	20 (83%)	4 (17%)

[a]Data expressed as mean ± S.E.M.
[b]No significant difference vs. Fas antigen–negative.
[c]$p < 0.01$ vs. CAH.

Expression of HCV Antigen (Core Antigen) and Fas Antigen Expression. Of the 40 samples tested for Fas antigen staining, 36 were immunostained for HCV antigen and 12 were positive (33%). Details of the expression of HCV antigen were reported previously (9). Hepatocytes with positive results for HCV antigen staining were scattered, and each positive cell was strongly stained in the cytoplasm (Fig. 1b). HCV-infected hepatocytes were found at or near the area with cells with Fas antigen expression among the infiltrating lymphocytes. Table 4 shows the relationship between Fas antigen expression and HCV antigen expression. Cells with Fas antigen expression were found in all samples but one, in which the HCV-infected hepatocytes were detected. The prevalence of Fas antigen expression in the HCV antigen–positive group (92%) was higher than that in the HCV antigen–negative group (58%) ($p < 0.05$).

TABLE 2. Semiquantitative analysis of Fas antigen expression and histological activity of liver inflammation

Area	Fas antigen expression (%)			
	–	+	++	+++
Periportal				
CPH (n = 16) (%)[a]	14 (87)	2 (13)	0 (0)	0 (0)
CAH (n = 24) (%)	8 (33)	8 (33)	7 (29)	1 (4)
Intralobular				
CPH (n = 16) (%)[b]	11 (69)	4 (25)	1 (6)	0 (0)
CAH (n = 24) (%)	7 (29)	14 (58)	3 (13)	0 (0)

Statistical analysis was made by means of χ^2 test.
[a]$p < 0.01$ vs. CAH.
[b]No significant difference vs. CAH.

TABLE 3. Periportal expression of Fas antigen and histological appearance

Fas antigen status	Periportal necrosis and inflammation	Lobular necrosis and inflammation	Portal inflammation
Fas antigen–positive (n = 18)	2.3 ± 0.4[a,b]	1.8 ± 0.3[c]	2.9 ± 0.1[d]
Fas antigen–negative (n = 22)	0.6 ± 0.2	1.5 ± 0.2	2.1 ± 0.2

Scoring was according to Knodell's HAI score.
[a]Data expressed as mean ± S.E.M.
[b]$p < 0.001$ vs. Fas antigen–negative.
[c]No significant difference vs. Fas antigen–negative.
[d]$p < 0.05$ vs. Fas antigen–negative.

DISCUSSION

Fas antigen is a mouse monoclonal antibody–defined cell surface protein that has an unknown physiological function, and anti-Fas can mediate apoptosis in cells with Fas antigen expression (20). In this study, Fas antigen was detected in hepatocytes from livers with HCV infection, but not in normal human liver tissue. In addition, more expression of Fas antigen was found in liver tissue with active inflammation than in that without it, particularly in the periportal area (Table 2), and the liver inflammation was more severe in the Fas antigen–positive group than in the Fas antigen–negative one (Table 3). These results indicate Fas antigen is an inducible molecule that can be expressed *de novo* in the liver tissue with HCV infection, and apoptosis mediated by Fas antigen may be one of the cell death patterns of HCV-infected liver tissue.

Hepatocytes with Fas antigen in HCV-infected liver were found particularly among the infiltrating lymphocytes. The hepatocytes that showed early changes of apoptosis have been also found to have lymphocytes closely applied to their surfaces (16), and these lymphocytes were revealed to be mainly composed of cytotoxic T lymphocytes (CTL) (27, 28). Some reports have shown that the death of CTL targets is apoptotic during viral infections, autoimmune reactions, and graft rejection (14), and cells treated with complement, lytic granules or perforin die by a necrotic process (29, 30). However, the mechanism of CTL delivery in an apoptosis-inducing signal remains unclear. TNF-related factors can induce apoptosis in tumor cells, but they are poor candidates for the mechanism of CTL-mediated DNA fragmentation, although Fas antigen shows structural homology with

TABLE 4. Relationship between Fas antigen expression and HCV antigen expression

HCV antigen status	Fas antigen–positive (%)
HCV antigen–positive (n = 12)	11/12 (92%)[a]
HCV antigen–negative (n = 24)	14/24 (58%)

[a]$p < 0.05$ vs. HCV antigen–negative.

TNF receptors. This is because TNF-related factors are slow-acting, inducing apoptosis in susceptible cells only after exposure of 24 hr or more, whereas CTLs can induce DNA fragmentation in minutes (31). It seems very likely that CTLs directly signal activation of a suicide program because CTLs can induce lysis and DNA fragmentation even under conditions that prevent exocytosis of CTLs (32-34). CTL probably interacts with the target cell by way of specific target cell membrane proteins including major histocompatibility complex molecules. Fas antigen may be one of these proteins, and the binding of CTLs to membrane proteins, including Fas antigen, might induce the cell to undergo apoptosis. Recently, a Fas ligand was found on the surface of a cytotoxic T-cell line of rat (35). The existence of CTLs is considered to be essential for the death by apoptosis of cells with Fas antigen expression, although no human Fas ligand has been detected.

Apoptosis but not classical necrosis of hepatocytes was found in the area of piecemeal necrosis by microscopic examination of biopsy samples from patients with CAH (16). At the light-microscopy level, however, apoptosis is inconspicuous because only the larger cell fragments (Councilman bodies) can be detected, and even these are

disposed of by the adjacent cells (granulocytes or macrophages) within a few hours (12, 15, 16). In this study, a large number of cells with Fas antigen was found among the infiltrating lymphocytes at the advancing edges of the so-called piecemeal necrosis in CAH. This supports the close relationship of apoptosis and the nature of the inflammatory infiltration extending into the parenchyma, which is regarded as characteristic of the activity in chronic hepatitis.

No significant difference in lobular inflammation was observed between the Fas-antigen–positive and negative groups. This is because the histological appearance of lobular inflammation is mainly evaluated for "necrosis" lesions (scattered foci of hepatocellular necrosis and ballooning degeneration) although short-lived acidophilic bodies (Councilman bodies) are also included. The result that Fas antigen expression has no correlation with the lobular inflammation might suggest that Fas antigen mediates not necrosis but apoptosis.

Fas antigen was found mainly in the cytoplasm of the hepatocytes and bile duct cells. There is a possible reason for the detection of Fas antigen in the cytoplasm, despite its being a cell-surface protein. Cells can die in the presence of *in vivo* ligand to the Fas antigen (anti-Fas–like cytokine) soon after the Fas antigen is expressed on the membrane, which may explain why Fas antigen expression is mainly found in the cytoplasm. Nerve growth factor receptor, the receptor family to which Fas antigen belongs, was reported to be detected by an immunohistochemical method, and positive decorations were found in the cytoplasm (36). This suggests the possibility of finding the surface protein in the cytoplasm by an immunohistochemical method. Fas antigen was expressed not only in the hepatocytes but also in the bile duct cells. Fas antigen can be expressed in bile duct cells during the process of the damage to these cells by HCV infection, which is known as one of the pathological features of chronic hepatitis C.

Sellins and Cohen (37) have shown that viral DNA in the host cell is cleaved along with cellular DNA by apoptosis—that is, a portion of the polyoma-virus DNA in transfected or infected cells suffers CTL-induced DNA fragmentation in parallel with the host cell DNA. In addition, CD4-positive and CD8-positive T cells with HIV infection were shown to die as a result of apoptosis (23, 38, 39), and anti-Fas was found to kill the chronically HIV-infected cells selectively. Thus viruses are known to induce apoptosis of their host cells, and some viruses themselves can be also cleaved. It is unknown what induces Fas antigen expression or apoptosis in hepatocytes of the liver with HCV infection, but this study showed that HCV-infected hepatocytes were found at or near the area with cells with Fas antigen expression among the infiltrating lymphocytes. This suggests that HCV infection of hepatocytes is closely related to the Fas antigen expression or apoptosis. HCV infection might trigger the induction of Fas antigen directly or through the immune system, and the infected cells might suffer CTL-induced DNA fragmentation. Further study is required to clarify this.

Fas antigen was expressed even in samples in which no HCV antigen was detected. There are two possible reasons for this. First, the sensitivity of the experiment for detecting the HCV antigen might not have been very good, that is, the HCV antigen might not have been detectable in the Fas antigen–expressed cells with HCV infection. Second, only HCV replication might be inhibited by the function of CTL in the Fas antigen–expressed cells that were alive among the infiltrating lymphocytes as CTL has been revealed to be able to inhibit HIV replication without inducing cell death (40). Further studies are necessary to examine these possibilities.

In this study, the role of apoptosis, which is one of the cell death patterns, the end point of liver injury, was investigated from the viewpoint of Fas antigen expression in trying to clarify the mechanism of HCV-related liver injury. Fas antigen expression (apoptosis) was concluded to play an important role in inflammation in the HCV-infected liver, especially the active inflammation of chronic hepatitis C.

REFERENCES

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359-362.
2. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989; 244:362-364.
3. Yuki N, Hayashi N, Hagiwara H, Takehara T, Oshita M, Kasahara A, Fusamoto H, et al. Serodiagnosis of chronic hepatitis C in Japan by second generation recombinant immunoblot assay. J Hepatol 1993;17:170-174.
4. Brown J, Dourakis S, Karayiannis P, Goldin R, Chiba J, Ohba H, Miyamura T, et al. Seroprevalence of hepatitis C virus nucleocapsid antibodies in patients with cryptogenic chronic liver disease. HEPATOLOGY 1992;15:175-179.
5. Garton JA, Tedder RS, Briggs M, Tuke P, Glazebrook JA, Trute A, Parker D, et al. Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. Lancet 1990;335:1419-1422.
6. Hagiwara H, Hayashi N, Mita E, Hiramatsu N, Ueda K, Takehara T, Yuki N, et al. Detection of hepatitis C virus RNA in chronic non-A, non-B liver disease. Gastroenterology 1992;102:692-694.
7. Weiner AJ, Kuo G, Bradley DW, Bonino F, Saracco G, Lee C, Rosenblatt J, et al. Detection of hepatitis C viral sequence in non-A, non-B hepatitis. Lancet 1990;335:1-3.
8. Takehara T, Hayashi N, Mita E, Hagiwara H, Ueda K, Katayama K, Kasahara A, et al. Detection of the minus strand of hepatitis C virus RNA by reverse transcription and polymerase chain reaction: implications for hepatitis C virus replication in infected tissue. HEPATOLOGY 1992;15:387-390.
9. Hiramatsu N, Hayashi N, Haruna Y, Kasahara A, Fusamoto H, Mori C, Fuke I, et al. Immunohistochemical detection of hepatitis C virus-infected hepatocytes in chronic liver disease with monoclonal antibodies to core, envelope and NS3 regions of the hepatitis C virus genome. HEPATOLOGY 1992;16:306-311.
10. Haruna Y, Hayashi N, Hiramatsu N, Takehara T, Hagiwara H, Sasaki Y, Kasahara A, et al. Detection of hepatitis C virus RNA in liver tissues by an in situ hybridization technique. J Hepatol 1993;18:96-100.
11. Walker NI, Harmon BV, Gobe GC, Kerr JFR. Patterns of cell death. Methods Achiev Exp Pathol 1988;13:18-54.
12. Searle J, Kerr JFR, Bishop CJ. Necrosis and apoptosis: distant modes of cell death with fundamentary different significance. Pathol Annu 1982;17:229-259.
13. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological

phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-257.
14. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.
15. Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol 1991;32:223-254.
16. Kerr JFR, Cooksley WGE, Searle J, Halliday JW, Halliday WJ, Holder L, Roberts I, et al. The nature of piecemeal necrosis in chronic active hepatitis. Lancet 1979;2:827-828.
17. Searle J, Harmon BV, Bishop CJ, Kerr JFR. The significance of cell death by apoptosis in hepatobiliary disease. J Gastroenterol Hepatol 1987;2:77-96.
18. Nakamura Y, Ohta G, Kono N, Kobayashi K, Kato Y. Electron microscopic observation of destruction of biliary epithelium in primary biliary cirrhosis. Liver 1983;3:238-248.
19. Weedon D, Searle J, Kerr JFR. Apoptosis: Its nature and implications for dermatopathology. Am J Dermatopathol 1979;1: 133-144.
20. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991;66:233-243.
21. Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA, Nagata S. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 1992;148:1274-1279.
22. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992;356: 314-317.
23. Kobayashi N, Hamamoto Y, Yamamoto N, Ishii A, Yonehara M, Yonehara S. Anti-Fas monoclonal antibody is cytocidal to human immunodeficiency virus-infected cells without augmenting viral replication. Proc Natl Acad Sci USA 1990;87:9620-9624.
24. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kierman TW, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. HEPATOLOGY 1981;1:431-435.
25. Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, et al. Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol 1991;65:1105-1113.
26. Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 1989;169: 1747-1756.
27. Russell JH. Internal disintegration model of cytotoxic lymphocyte-induced target damage. Immunol Rev 1983;72:97-118.
28. Takehara T, Hayashi N, Katayama K, Kasahara A, Fusamoto H, Kato M, Masuzawa M, et al. Two-dimensional flow cytometric analysis of intrahepatic lymphocyte subsets from patients with chronic hepatitis. Dig Dis Sci 1991;36:87-91.
29. Henkart PA. Mechanism of lymphocyte-mediated cytotoxicity. Annu Rev Immunol 1985;3:31-58.
30. Young JD, Cohn ZA. Cell-mediated killing: a common mechanism? Cell 1986;46:641-642.
31. Cohen JJ, Duke RC. Apoptosis and programmed cell death in immunity. Annu Rev Immunol 1992;10:267-293.
32. Ostergaard HL, Clark WR. Evidence for multiple lytic pathways used by cytotoxic T lymphocytes. J Immunol 1989;143:2120-2126.
33. Ostergaard HL, Kane KP, Mescher MF, Clark WR. Cytotoxic T lymphocyte mediated lysis without release of serine esterase. Nature 1987;330:71-72.
34. Trenn G, Takayama H, Sitkovski MV. Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. Nature 1987;330:72-74.
35. Suda T, Takahashi T, Gobstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993;75:1169-1178.
36. Taniguchi A, Clark HB, Johnson EM. Induction of nerve growth factor in Schwann cells after axotomy. Proc Natl Acad Sci USA 1986;83:4094-4098.
37. Sellins KS, Cohen JJ. Polyomavirus DNA is damaged in target cells during cytotoxic T-lymphocyte-mediated killing. J Virol 1989;63:572-578.
38. Meyaard L, Otto SA, Jonker RR, Mijnster MJ, Keet RPM, Miedema F. Programmed death of T cells in HIV-1 infection. Science 1992;257:217-219.
39. Laurent-Crawford AG, Krust B, Muller S, Riviere Y, Rey-Cuille MA, Bechet JM, Montagnier L, et al. The cytopathic effect of HIV is associated with apoptosis. Virology 1991;185:829-839.
40. Martz E, Howell DM. CTL: virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. Immunol Today 1989;10:79-86.

0023-6837/93/6904-0415$03.00/0
LABORATORY INVESTIGATION

Vol. 69, No. 4, p. 415, 1993
Printed in U.S.A.

Constitutive and Induced Expression of APO-1, A New Member of the Nerve Growth Factor/ Tumor Necrosis Factor Receptor Superfamily, In Normal and Neoplastic Cells

FRANK LEITHÄUSER, JENS DHEIN, GUNHILD MECHTERSHEIMER, KARIN KORETZ, SILKE BRÜDERLEIN, CHRISTOF HENNE, ANNETTE SCHMIDT, KLAUS-MICHAEL DEBATIN, PETER H. KRAMMER, AND PETER MÖLLER

Department of Pathology, University of Heidelberg, and Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, Heidelberg, Germany

BACKGROUND: APO-1 is a 48 kilodalton transmembrane, cysteine-rich glycoprotein identical with the Fas antigen which belongs to the nerve growth factor/tumor necrosis factor receptor superfamily. Cross-linking of APO-1 induces apoptotic cell death in sensitive cells.

EXPERIMENTAL DESIGN: As suggested by our preliminary results, APO-1 expression is not restricted to cells of the hematopoietic lineage. We therefore investigated APO-1 expression in normal human tissues and in various epithelial and nonepithelial tumors.

RESULTS: We show by immunohistochemistry that APO-1 is a non-lineage antigen constitutively expressed in a variety of epithelial cells. This includes the basal layers of various squamous epithelia, transitional epithelium and columnar epithelium of the biliary tract and intestine. Among the epithelial cell types of the reproductive system of both genders, APO-1 expression is complex. Except the satellite cells of autonomic ganglia, all cells of the nervous tissue are APO-1-negative. Among mesenchymal cells, constitutive APO-1 expression is rare but detectable in various kinds of activated cells, *e.g.* fibroblasts, osteoblasts, and subpopulations of endothelial cells. Within the immune system, APO-1 is broadly distributed among histiocytic cells but restricted to minor subpopulations of peripheral T and B cells. Immature T cells, *i.e.*, thymocytes, do not express detectable APO-1-antigen. Expression of APO-1 was induced in phytohemagglutinin activated T cells and in a mammary carcinoma cell line by interferon-γ alone and in combination with tumor necrosis factor α. Consistently, there was an *in situ* induction of APO-1 in several types of glandular pithelium in microtopographic association with lymphohistiocytic infiltrates. This inflammation-associated APO-1 induction went along with increased expression of this molecule within the lymphocytic compartment of the lesion. In tumors, APO-1 expression was heterogeneous. In comparison to their normal counterparts, some tumors showed abnormal hypo-expression or loss of APO-1. However, abnormal neo-expression was also found.

CONCLUSIONS: Tissue distribution, *in vitro* expression, and reaction upon cytokine-induced activation suggest that APO-1 might not only transmit apoptotic signals but might play a more general role in growth control.

Additional key words: Fas-Antigen, Tissue distribution.

We previously described a 48 kilodalton cell membrane protein, APO-1, recognized by the monoclonal antibody anti-APO-1. APO-1 is expressed on human T cell leukemia virus type 1 transformed T cell lines, cultured cells from patients with adult T cell leukemia, activated normal T and B cells, Epstein Barr virus (EBV) transformed lymphoblastoid B cell lines (LCL) and on some B lymphoma lines (1–3). Cross-linking of APO-1 with anti-APO-1 induces apoptosis in APO-1 positive, apoptosis sensitive cells *in vitro* (4). *In vivo*, anti-APO-1 induced tumor regression of human LCL tumors in *nu/nu* and severe combined immunodeficiency (SCID) mice by apoptosis (1, 4). The monoclonal antibody anti-Fas, previously described by Yonehara *et al.* (5), has a similar *in vitro* activity as anti-APO-1. Cloning of the APO-1 and the Fas antigen revealed that both mAb recognize the same antigen (6, 7). The APO-1 antigen is a cysteine-rich transmembrane glycoprotein of 335 amino acids and

belongs to the NGF/TNF receptor superfamily. The human APO-1 gene is located on 10q23 (8). The physiologic ligand of APO-1/Fas is still unknown. Recently, the lymphoproliferative disorder in *lpr/lpr* mice was traced to a mutation in the gene encoding the murine Fas/APO-1 antigen (9). *lpr/lpr* mice show an abnormal accumulation of $CD4^-/CD8^-$ peripheral T cells and high autoantibody titers associated with autoimmune disease reminiscent of systemic lupus erythematosus. These effects might be due to a defect in apoptosis of T and B cells caused by a lack of Fas/APO-1 (10).

EXPERIMENTAL DESIGN

Our own preliminary experiments suggested a broad APO-1 distribution in normal cells and tissues. In addition, anti-Fas antibody was shown to have lytic effects not only on the histiocytic cell line U937 and the promyelocytic leukemic cell line HL60 but also on the rhabdomyosarcoma cell line A673 and—in combination with interferon-γ IFN-γ—even on the colon carcinoma cell line HT29 (5). Thus, we concluded that APO-1/Fas expression is not restricted to cells of the hematopoietic lineage. We therefore investigated APO-1 expression in normal human tissues and in a representative collection of epithelial and mesenchymal tumors. The original anti-APO-1 antibody is of the IgG3 isotype. Monoclonal antibodies of IgM, IgG2a, IgG2b, and IgG3 isotype may produce undesired side reactions in immunohistochemistry, especially in frozen tissue preparations. However, undesired side reactions are minimal for IgG1 antibodies. Therefore, we used an IgG1 switch variant of anti-APO-1 (4) to assess APO-1 expression in tissues.

RESULTS AND DISCUSSION

APO-1 Expression in Normal Adult Tissues.

The detailed data on *in situ* expression of APO-1 in normal cell types are given in Tables 1 and 2.

Among epithelial cell types, APO-1 expression was heterogeneous. All types of squamous epithelium showed APO-1 at high density at the cytomembrane of the basal layer (Fig. 1, 4*F*). Suprabasal layers showed low APO-1 density. Cells of the stratum granulosum of all sites and the cornified layers of the epidermis and the tongue were APO-1-negative. Thymic epithelium (Fig. 5*A*) was APO-1-positive, except for a subpopulation of cortical epithelial cells. Epithelial cells of the dermal adnexae (Fig. 2) and histogenetically related glands showed a differential APO-1 expression. Myoepithelial cells were positive in dermal glands and predominantly positive in the mammary gland (Fig. 10*A* and *B*) but constitutively negative in serous and salivary glands (Fig. 9*B*). Apocrine cells of all sites were negative. Except for a minor subpopulation of pancreatic acinar cells all types of secretory gland epithelia were entirely APO-1-negative under normal conditions (Fig. 9*B*). All epithelial components of the gastric mucosa (Fig. 3*F*), duodenal glands and Paneth cells were devoid of APO-1. By contrast, intestinal epithelia expressed APO-1 at high density within the cytoplasm and at their basolateral cell surface (Fig. 3*B*). The same was true for the cuboid epithelium of the bile ducts (Fig. 3*D*), the gallbladder (Fig. 3*C*), the mucociliar epithelia of the respiratory tract (Fig. 3*A*), and for the ciliated and nonciliated cells of the fallopian tube (Fig. 4*D*). Alveolar epithelium was faintly positive at its basal site (Fig. 3*A*). Along the nephron, APO-1 expression was restricted to the proximal and the collecting tubules (Fig. 3*E*). Transitional epithelium of different sites (upper respiratory tract and urinary tract) was APO-1 positive in the basal layers and negative in the cells reaching the surface. In the prostate, intense and consistent APO-1 staining was found at the basal cells, in contrast with the absence of the antigen among the mature epithelial cells lining the alveolar lumen (Fig. 4*G*). Male and female germ cells were clearly APO-1 negative (Fig. 4*A* and *C*), as were the Sertoli cells within the seminiferous tubules (Fig. 4*C*), the follicular epithelium of the ovarian primary follicle (Fig. 4*A*) and the granulosa cells of the ovulating follicle (Fig. 4*B*). Epithelia of endocrine organs, pancreatic islands included, were APO-1-negative except for adrenal cortex epithelium which expressed APO-1 in all three zones (Fig. 3*F*). In the pituitary gland, the epithelium of cysts within the pars intermedia was the only APO-1-positive type of epithelial cell (these, however, are not part of the endocrine system). By contrast, the argyrophilic (APUD) cells in respiratory and gastrointestinal mucosae lacked detectable APO-1. Testicular Leydig cells (Fig. 4*C*) and the ovarian theca cells (Fig. 4*B*) were APO-1-positive. As a peculiar feature, the columnar cells of the ductus epididymidis testis (Fig. 4*E*) were markedly heterogeneous, expressing APO-1 in a seemingly random-like fashion. Irrespective of cyclic changes (detailed data not shown), the endometrial glands were APO-1-positive. The endometrial stromal cells were constitutively APO-1-negative but expressed this molecule once transformed into chorionic cells.

Neuronal and glial cells of the central nervous system and cells constituting peripheral nervous tissues were generally devoid of APO-1 (Fig. 8*A* and *B*) with the only exception of satellite cells surrounding peripheral ganglia. These cells expressed APO-1 at low levels (Fig. 8*B*). APO-1 was undetectable within a traumatic neuroma (which is a reactive proliferation of peripheral neurits together with Schwann cells). Among adult mesenchymal cell types APO-1 expression was scarce. The molecule was regularly present in/on osteoblasts, renal mesangial cells (Fig. 3*E*), and vascular smooth muscle cells. While resting fibrocytes (*e.g.*, in tendons) were APO-1 negative, fibroblasts of healing wounds and fresh scars (Fig. 7*A*) did express APO-1. Lymphatic, venous and arterial endothelium was APO-1-negative under normal conditions as were endothelial cells of the smaller blood vessels, epithelioid venules included (Fig. 5*C*). However, we found APO-1 consistently present in capillaries of the brain (Fig. 8*A*) and in the fetal part of the placenta. APO-1 expression in capillary endothelium was seen in only one specimen of skeletal muscle and thus seems to be rare (Fig. 12*B*) in normal, non-inflamed adult tissue.

APO-1 expression within cells of the immune system was complex. Cortical and medullary thymocytes did not show APO-1 expression (Fig. 5*A*). Flow cytometry studies of purified thymocytes revealed only negative cells (Fig. 6*A*). In peripheral lymphoid tissues, a small subset of T cells in T areas was APO-1-positive (Fig. 5*B* and

TABLE 1. EXPRESSION OF APO-1 ON NORMAL EPITHELIAL CELLS AS DETERMINED BY IMMUNOHISTOCHEMISTRY

Skin and dermal appendages	
Epidermis	
Melanocytes	−
Keratinocytes	
Stratum basale	+
Stratum spinosum	(+)
Stratum granulosum	−
Stratum corneum	−
Hair	
Shaft	−
External root sheath	+
Internal root sheath	
Lower part	−
Upper part	+
Cortical substance and cuticle	−
Cortex and outer layers	+
Sebaceous glands	
Acinar cells	+
Ductal cells	+
Sweat glands	
Eccrine	
Acinar cells	− > +
Ductal cells	−
Apocrine (axillary, ceruminous)	
Acinar cells	−
Ductal cells	−
Myoepithelial cells	+
Lacrimal gland	
Glandular cells	−
Ductal cells	−
Mammary gland	
Acinar cells[a]	±
Ductal cells[a]	±
Myoepithelial cells	+ > −
Thymus	
Subcapsular epithelium	+
Medullary epithelium	+
Cortical epithelium	±
Gastrointestinal tract	
Major salivary glands	
Mucous acinar cells[a]	−
Serous acinar cells[a]	−
Intercalated duct cells[a]	−
Striated duct cells[a]	−
Excretory duct cells[a]	−
Myoepithelial cells[a]	−
Minor mixed salivary glands	
Mucous acinar cells	−
Serous acinar cells	−
Ductal cells	−
Tongue	
Stratified squamous epithelium	
Stratum basale	+
Stratum spinosum	(+)
Stratum granulosum	−
Stratum corneum	−
Urinary tract	
Kidney	
Bowman's capsule	−
Proximal tubule	+
Distal tubule	−
Loop of Henle (thin segment)	−
Collecting tubule	+
Efferent urinary passages	
Transitional epithelium	
Basal layer	+
Superficial layer	−
Endocrine system	
Hypophysis	
Pars distalis	
Acidophils	−
Basophils	−
Chromophobes	−
Pars intermedia	
Cyst epithelium	+
Thyroid gland	
Thyroid epithelial cells	−
Parafollicular C-cells	−
Parathyroid gland	
Chief cells	−
Oxyphilic cells	−
Adrenal cortex	
Zona glomerulosa	+
Zona fasciculata	+
Zona reticularis	+
Adrenal medulla	
Adrenal chromaffine cells	−
Male reproductive system	
Testis	
Germ cells	−
Spermatogonia	−
Primary spermatocytes	−
Secondary spermatocytes	−
Spermatids	−
Spermatozoae	−
Sertoli cells	−
Leydig cells	+
Excretory ducts of the testis	
Rete testis	+
Ductus epididymidis	
Basal layer	+
Columnar cells	− > +
Ductus deferens	
Basal cells	+
Columnar cells	+
Prostate gland	
Glandular cells	−
Ductal epithelium[a]	−
Basal cells	+
Tonsil	
Surface epithelium	
Stratum basale	+
Stratum spinosum	(+)
Stratum granulosum	−
Crypt epithelium	+
Esophagus	
Stratified squamous epithelium	
Stratum basale	+
Stratum spinosum	(+)
Stratum granulosum	−
Stomach	
Surface mucous cells[a]	−
Chief cells	−
Parietal cells	−
Mucous neck cells	−
Pyloric gland cells	−
Endocrine cells	−

continued

TABLE 1. *Continued*

Duodenum	
Villous epithelial cells	+
Goblet cells	+
Crypt cells	+
Brunner's glands	−
Mucous neck cells of Brunner's glands	−
Paneth cells	−
Jejunum, ileum, colon	
Villous epithelial cells	+
Goblet cells	+
Crypt cells	+
Pancreas	
Acinar cells	− > +
Centroacinar cells	−
Ductal cells	−
Islet cells	−
Liver, gallbladder	
Hepatocytes	+
Bile duct epithelium	+
Gallbladder epithelium	+
Respiratory tract	
Larynx	
Stratified squamous epithelium	
Stratum basal	+
Stratum spinosum	(+)
Stratum granulosum	−
Transitional epithelium of laryngeal ventricle	
Basal layer	+
Superficial layer	−
Trachea, Bronchus	
Ciliated cells	+
Goblet cells	+
Alveolar cells	(+)
Female reproductive system	
Ovary	
Oocytes	−
Follicular epithelium	−
Theka folliculi	+
Fallopian tube	
Ciliated cells	+
Nonciliated cells	+
Uterus	
Endometrial gland cells[b]	+
Stromal cells	−
Chorionic epithelium	+
Cervix uteri	
Columnar epithelium	+
Mucous gland cells	−
Vagina	
Stratified squamous epithelium	
Stratum basal	+
Stratum spinosum	(+)
Placenta	
Trophoblast	−
Amnion cells	+

[a] Positive in areas of chronic inflammation.

[b] Irrespective of cyclic changes.

Staining intensity: + positive; (+) intermediate; ± positive and negative cells in about equal proportions; + > − more positive than negative cells; − > + more negative than positive cells; − negative.

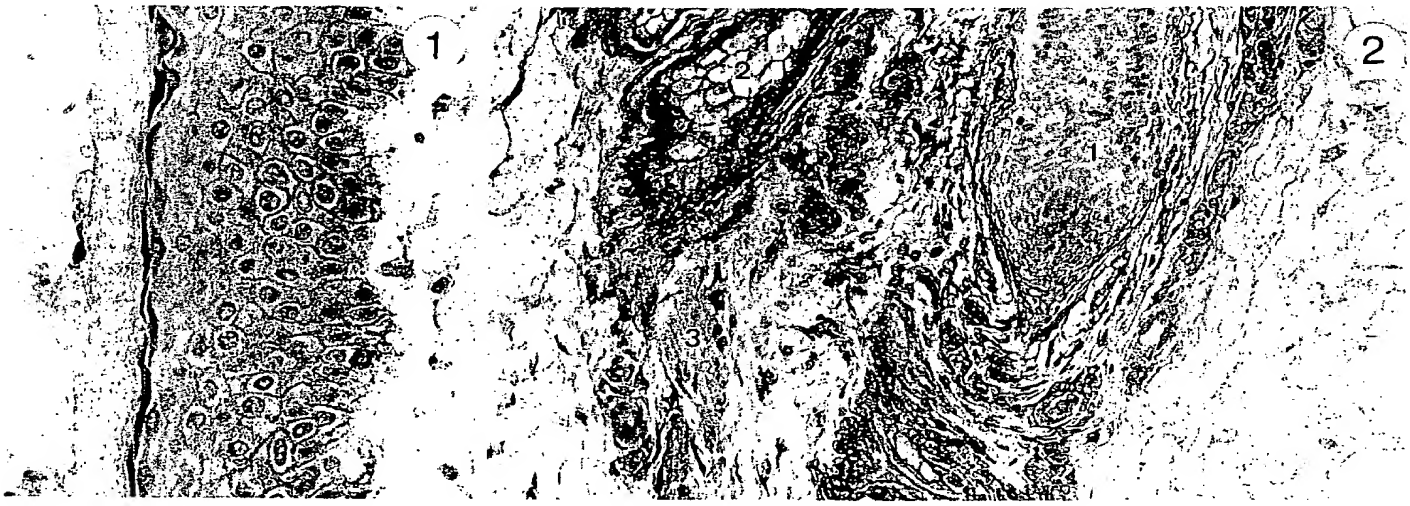

FIG. 1. Epidermis. APO-1 positivity of the stratum basale and progressive loss of antigen expression towards the epidermal surface. ×343.

FIG. 2. Dermal appendages. No anti-APO-1 staining can be detected in the lower part of the internal root sheath of the hair (*1*) and within the arrector pili muscle (*3*). The sebaceous glands (*2*) strongly express APO-1. Positive staining in connective tissue surrounding the hair root is due to APO-1 expressing fibroblasts. ×43.

TABLE 2. EXPRESSION OF APO-1 WITHIN NORMAL MESENCHYMAL AND NERVAL AND LYMPHOID TISSUE AS DETERMINED BY IMMUNOHISTOCHEMISTRY

Nerval tissue	
Central nervous system	
Neural cells	
Cerebral cortex	−
Cerebellar cortex	−
Pons cerebri	−
Neurohypophysis	−
Neuroglia	
Astroglia	−
Oligodendroglia	−
Microglia	−
Ependyma	−
Peripheral nervous system	
Peripheral ganglion cells	−
Satellite cells	+
Normal and regenerating peripheral nerve	
Axon	−
Schwann cells	−
Adrenal medulla	−
Enterochromaffin cells	−
Peripheral nerve endings	
Corpuscule of Vater-Pacini	−
Meissner's corpuscle	−
Mesenchymal tissue	
Connective tissue	
Fibroblasts	±
Fibrocytes	−
Chondrocytes	−
Osteoblast	+
Adipocytes	−
Endothelial cells	
CNS, placenta, skeletal muscle	±
All other organs	−
Mesangial cells	+
Muscle tissue	
Skeletal muscle	−
Cardiac muscle	−
Smooth muscle	
Tunica media of blood vessels	+
All other sites	−
Immune cells	
T cells	
Thymic cortex	−
Thymic medulla	−
Splenic periarteriolar lymph sheath	− ≫ +
Nodal T-area	− ≫ +
B cells	
Mantle zone	−
Follicle center	−/(+)
Extrafollicular B-region	−
Tonsillar intraepithelial B cells	−
Nodal sinusoidal B cells	+
Thymic medullary B cells	−
Plasma cell compartment	−
Histiocytic and related cells	
Interstitial dendritic cells	−
Follicular dendritic cells	
Apical	+
Basal	−
Sinus histiocyte/lining cells	+
Starry sky macrophages	+
High endothelium of venules	+
Foreign body giant cells	+
Foamy cells	±
Kupffer cells	+
Osteoclasts	−
Placental Hofbauer cells	−

Staining: + positive; (+) intermediate; ± positive and negative cells in about equal proportions; + > − more positive than negative cells; − > + more negative than positive cells; − negative.

C). This finding again, was supported by flow cytometry. Tonsillar T cells consisted of two subpopulations, a major APO-1-negative subset and a smaller subset displaying weak APO-1-positivity. Upon *in vitro* activation with phytohemagglutinin, nearly all T cells expressed APO-1 (Fig. 6*B*). During peripheral B lymphocyte ontogeny, APO-1 expression was detected at low levels in a major subpopulation of follicle center B blasts (Fig. 5*B*). These cells were located in the basal, dark zone of follicle centers and could be clearly distinguished from follicular dendritic cells that were devoid of APO-1 at this site. In the apical, light zone of the follicle center, the strongly stained dendritic network prevented the assessment of APO-1 expression of follicular center B cells (Fig. 5*D*). Mantle zone B lymphocytes and mature plasma cells were unstained in all sites examined. Epithelium-associated B cells that morphologically resemble marginal zone B cells of the spleen and small, cleaved follicle center B cells in the mucosa-associated lymphoid tissues and that have small dendritic cytoplasmic extensions within the thymic medulla, lacked APO-1 (Fig. 5*A* and *D*). By contrast, sinusoidal (*syn.* monocytoid) B cells giving rise to the so-called sinusoidal B cell reaction (as seen in toxoplasmic lymphadenitis and lymphadenopathy due to early HIV infection (11, 12)) expressed APO-1 at high levels. With the exception of osteoclasts and placental Hofbauer cells, APO-1 was broadly expressed at high levels by a large majority of histiocytic cells. The follicular dendritic cells were APO-1-negative in the basal (so-called dark zone) and APO-1-positive in the apical part (so-called light zone) of the follicular dendritic network (Fig. 5*D*). In addition, foamy cells as a special variant of phagocytes showed a heterogeneous pattern of APO-1 expression.

We also observed induction of APO-1 expression in several types of constitutively APO-1 negative epithelia. Induction was microtopographically related to chronic, lymphohistiocytic inflammation. Inflammation-associ-

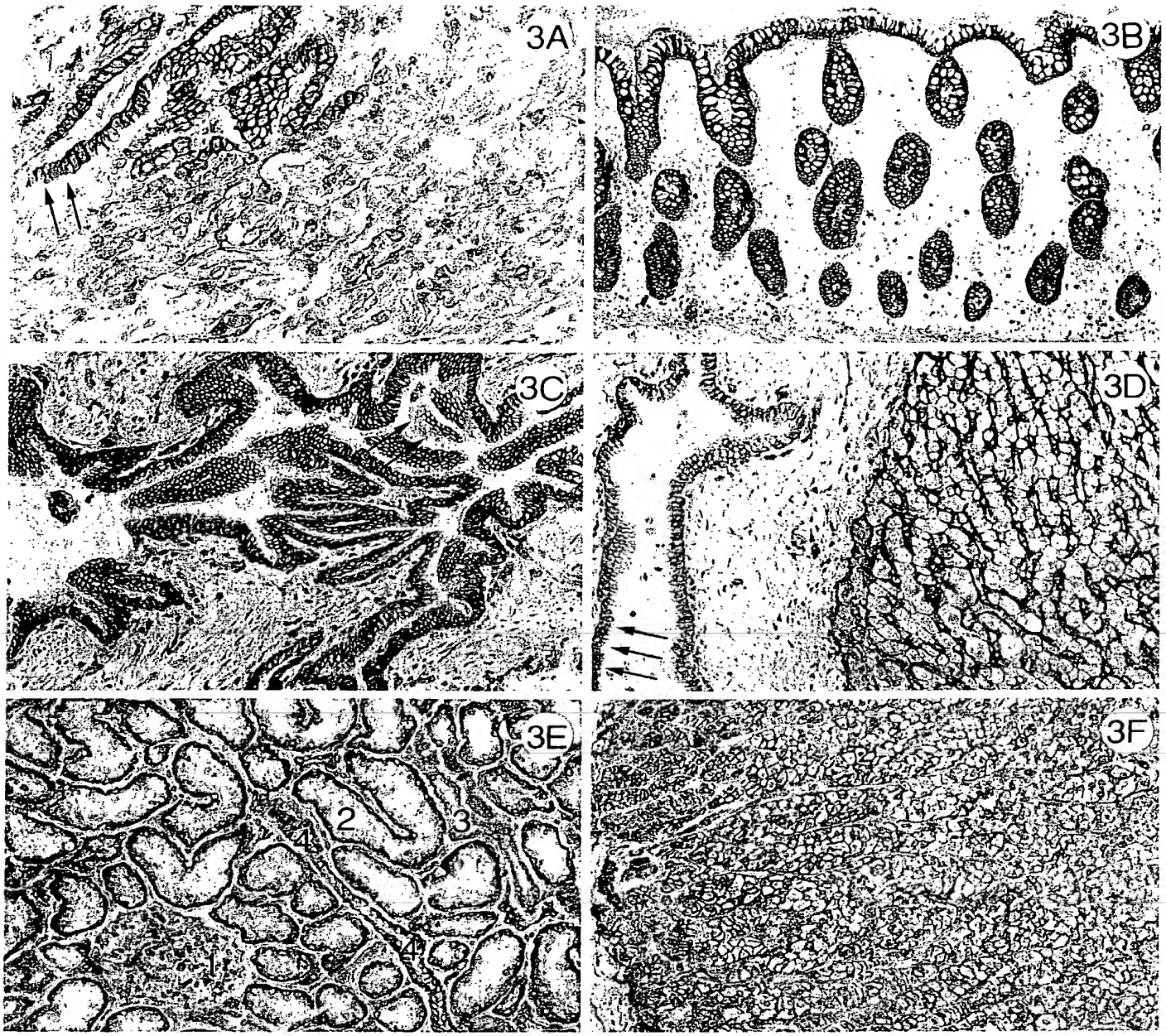

FIG. 3. APO-1 expression in the respiratory and gastrointestinal tract and adrenal gland. *A*, Lung. Faint staining of the alveolar epithelium. The epithelial lining cells of a respiratory bronchus are APO-positive on the basal and lateral surfaces while no anti-APO-1 binding is detectable on the luminal side (*arrows*). *B*, Colon. Intense basolateral and cytoplasmatic APO-1 expression of the mucosal epithelium cells irrespective of cell type or location within the crypt. *C*, Gallbladder. The columnar epithelium is APO-1 positive on the basal and lateral and negative on the luminal cell surfaces. Arrows indicate a tangentially hit luminal epithelial surface showing no staining with anti-APO-1. *D*, Liver. Hepatocytes are consistently APO-1 positive. The bile duct epithelium expresses APO-1 on its basal and lateral cell surfaces, whereas the luminal cytomembrane (*arrows*) is devoid of the antigen. *E*, Renal cortex. Glomeruli (*1*) including visceral and parietal leave of Bowman's capsule and the distal tubules are mainly unstained with only the mesangial cells expressing APO-1. Proximal (*3*) and collecting tubules (*4*) are consistently APO-1 positive. *F*, Adrenal gland. All epithelial layers of the adrenal cortex express APO-1. Figure 3*A*, ×257; *B*, ×64; *C*, ×129.

ated APO-1 induction was found in glandular and ductal epithelium of the salivary gland (Fig. 9*B*), the mammary gland, pancreas, the prostate (Fig. 9*A*), and in the surface mucus cells of the stomach. Induced APO-1 expression was not confined to epithelial cells, but also included the inflammatory lymphocytes themselves (Fig. 9*A* to *C*). Furthermore, normal resting mesothelium was APO-1 negative. By contrast, in inflammatory effusions activated and proliferating mesothelial cells were mainly APO-1-positive (Fig. 7*B*). However, chronic inflammation did not always induce APO-1 expression in epithelial cells: Figure 9*C* illustrates a chronic orchitis with APO-

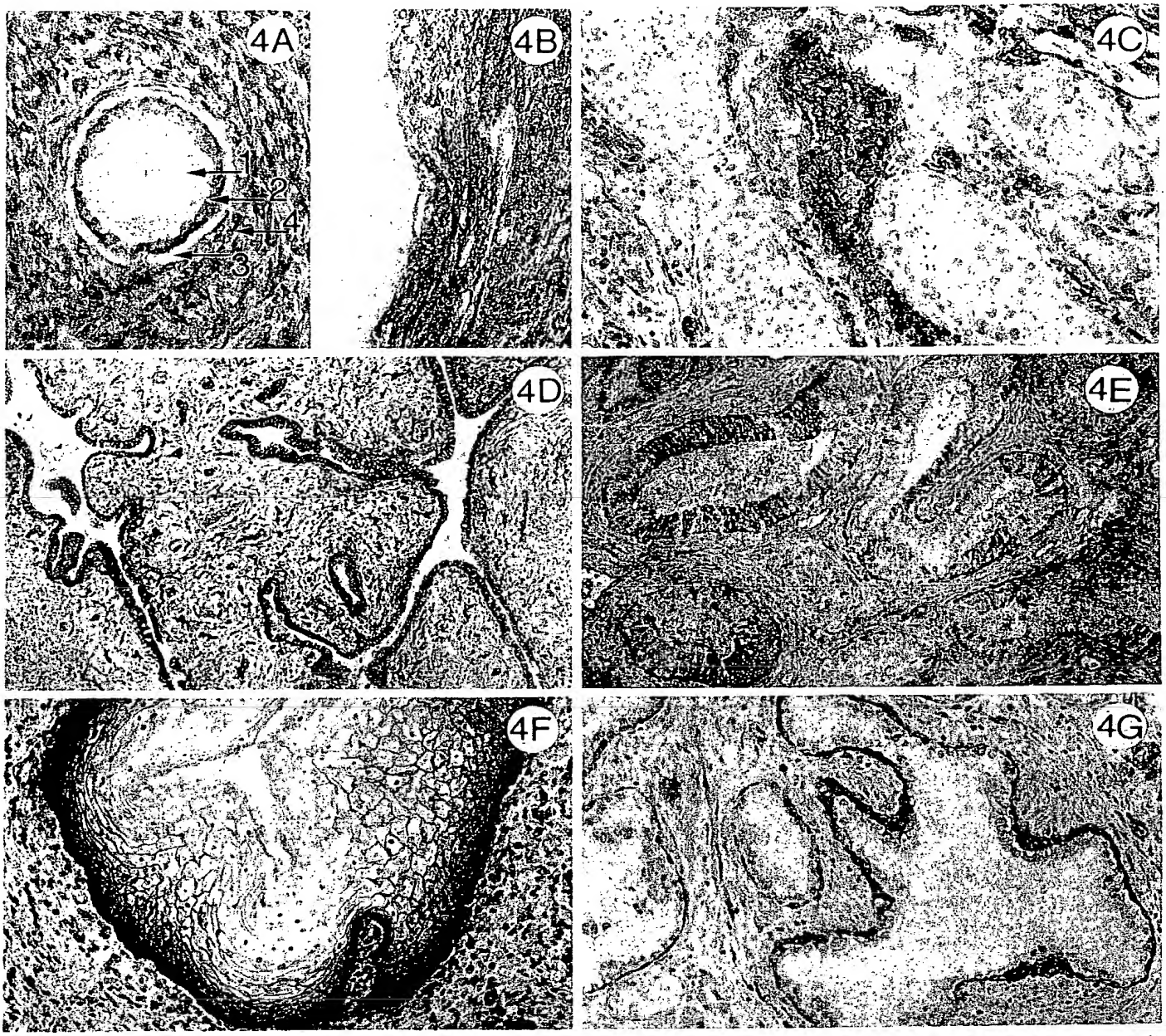

FIG. 4. APO-1 expression within the male and female reproductive system. *A*, Ovary (primary follicle). Oocyte (*1*) and basal membrane (*3*) reveal no APO-1 expression, whereas the ovarian stroma (*4*) is APO-1 positive. Apparent staining of the actually APO-1 negative follicular epithelium (*2*) is caused by the dense chromatin of cell nuclei counterstained with hematoxylin. *B*, Ovary (ovulating follicle). The granulosa cells are APO-1 negative while the thecal cells display intense antigen expression. *C*, Testicle. No APO-1 expression can be detected on the germ cells and Sertoli cells within the seminiferous tubules but Leydig cells of the interstitium are APO-1 positive. *D*, Fallopian tube. The mucosal lining is composed of markedly APO-1 positive epithelial cells. *E*, Epididymis. Consistent APO-1 expression is displayed by the basal cells as compared with a heterogeneous distribution of the antigen among the columnar cells. *F*, Vagina. Stratified, uncornified epithelium showing a continuous loss of APO-1 expression from the markedly positive basal to the clearly negative luminal cell layer. *G*, Prostate gland. No APO-1 antigen is expressed in the alveolar epithelium, whereas the basal cells exhibit intense anti-APO-1 staining. Stromal smooth muscle cells and fibrocytes are APO-1 negative. Figure 4*A*, ×136; *B*, ×100; *C*, *F*, and *G*, ×129; *D* and *E*, ×64.

1 negative germinal epithelium and Sertoli cells in the vicinity of a lymphocytic infiltrate.

Induction of APO-1 in inflammation in vivo may be mediated by cytokines. This is supported by experiments using the MCF-7 mammary carcinoma cell line. MCF-7 shows weak APO-1 expression (Fig. 11*A*). APO-1 expression was stimulated by IFN-γ, TNF-α or the combination of both cytokines. Fig. 11*B* shows APO-1 induction by TNF-α in a minor subset of MCF-7 cells. Considerably stronger induction of APO-1 was seen with IFN-γ (Fig. 11*C*). Interferon induced APO-1 expression was only slightly weaker than APO-1 expression induced by both cytokines in combination (Fig. 11*D*). These findings are in line with data by Itoh *et al.* (6).

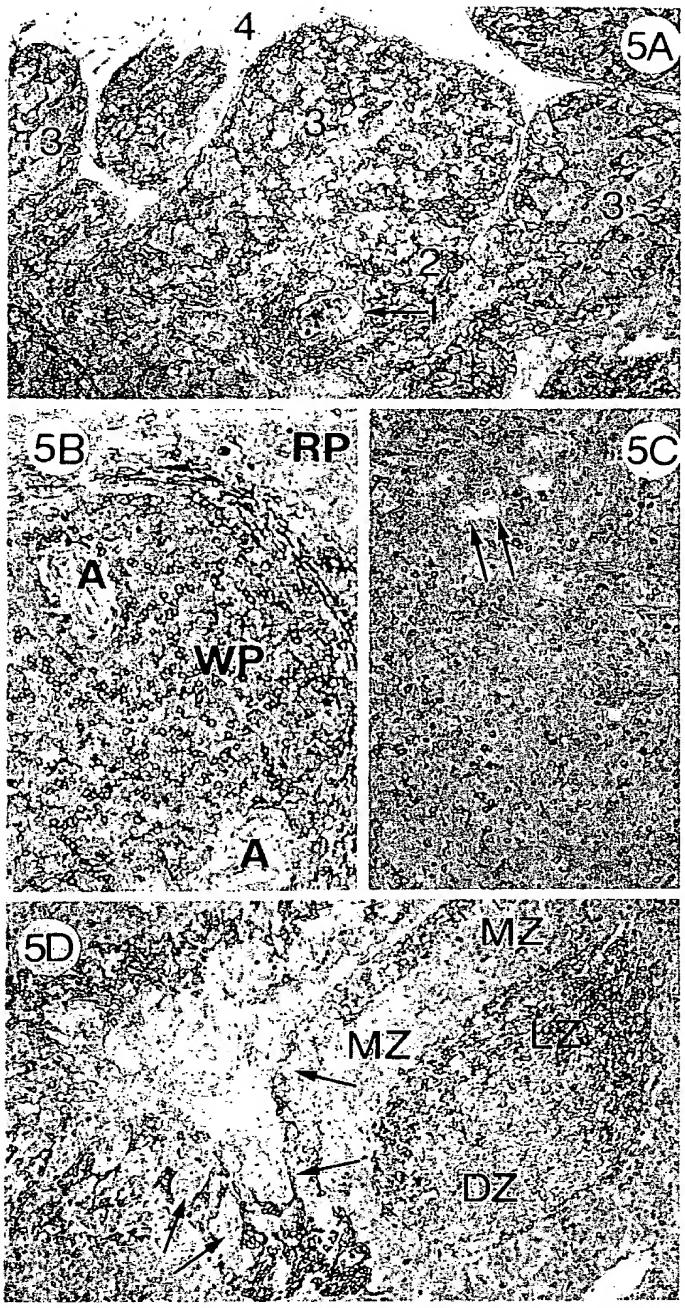

FIG. 5. Expression of APO-1 within the lymphatic tissue. *A*, Thymus. The T lymphocytes of the cortex (*3*) and the medulla (*2*) are devoid of any detectable APO-1. Positive staining within these areas is due to APO-1 positive subcortical and medullary epithelium and the heterogeneously APO-1 expressing cortical epithelium. A Hassall's corpuscle (*1*) and the trabecular connective tissue (*4*) are largely unstained. *B*, Spleen. Two central arteries (*A*) are surrounded by white pulp (*WP*) consisting of an admixture of APO-1-positive and -negative T lymphocytes. The red pulp (*RP*) shows no detectable APO-1 expression. *C*, Lymph node (T-zone). Only a small subset of T lymphocytes exhibits APO-1 expression. The *arrows* indicate postcapillary venules lined by APO-1 negative endothelium. *D*, Tonsil. APO-1 is strongly expressed on crypt epithelium. Intraepithelial lymphoid cells and lymphocytes of the follicular mantle (*MZ*) are APO-1-negative. The B blasts of the follicle center are weakly APO-1-positive. The strongly stained cells are dendritic cells of the light zone (*LZ*) of the follicle center, whereas the dendritic network of the dark zone (*DZ*) is APO-1-negative. Figure 5*A*, ×64; *B* and *D*, ×129; *C*, ×100.

APO-1 EXPRESSION IN TUMORS

The data on APO-1 expression in tumors as determined by immunohistochemistry are given in Table 3.

Four patterns of APO-1 expression in tumors were observed. *Pattern 1.* Both tumor cells and the normal cellular counterparts express APO-1 or are both APO-1-negative. This pattern was most often observed in benign tumors, *e.g.*, colorectal adenomas, neurilemmomas, neurofibromas, and fibromas. *Pattern 2.* In comparison to APO-1-positive normal cells, the corresponding tumor cells are APO-1-negative or only weakly positive. This pattern was predominantly found in carcinomas, *e.g.*, lung carcinoma and colorectal carcinoma. *Pattern 3.* Tumors derived from APO-1 negative normal cells neoexpress APO-1. This pattern was quite often found in (malignant) mesenchymal tumors, *e.g.*, in leiomyomas, leiomyosarcomas, malignant schwannomas, and in liposarcomas. *Pattern 4.* As an extension of pattern no. 3, a subgroup of tumors could be identified that may show oncofetal regulation of APO-1 expression, *i.e.*, the fetal cell type is APO-1-positive, adult normal cells are APO-1-negative and tumor cells re-express APO-1. This pattern was observed, *e.g.*, in a rhabdomyosarcoma (Fig. 12*A* to *C*). In cell types in which APO-1 expression is transient and regulated by external stimuli, *e.g.*, in B lymphocytes, APO-1 expression in the respective neoplasias is likely to be complex. This was indeed the case in B cell leukemias and lymphomas. Taken together, the data on APO-1 expression in tumors suggest that neoplastic transformation leads to either retention, loss or neoexpression of APO-1. Retention of physiologic levels and loss and gain of APO-1 expression is also observed among tumors of the same category, *e.g.*, breast carcinoma (Fig. 10*B*). Anomalous APO-1 expression expression is more frequently found in malignant tumors as compared with benign proliferations. Abnormal expression of APO-1 in neoplastic clones might have substantial consequences for the clinical course of the disease.

THE POSSIBLE ROLE OF APO-1 IN APOPTOSIS AND GROWTH CONTROL *IN VIVO*

Both anti-APO-1 and anti-Fas were initially described as monoclonal antibodies capable of killing cells *in vitro* and *in vivo* (1, 5). The mode of killing was different from antibody-mediated, complement-dependent lysis. *In vitro* (and probably also *in vivo*), anti-APO-1-mediated cell death was shown to be due to cross-linking of APO-1 molecules on the target cell (4). Anti-APO-1 induced cell death corresponded to current criteria of apoptosis (13, 14). Ultrastructural analysis of anti-APO-1 effects on the SKW6.4 lymphoblastoid B cell line revealed nuclear invaginations 15 minutes and chromatin condensation 30 minutes after incubation. Three hours after onset of incubation, all cells were totally desintegrated (15). APO-1 was therefore considered a receptor for an apoptotic signal. This assumption is supported by experiments of Itoh *et al.* (6) and Oehm *et al.* (7). These authors observed that mouse L929 fibroblasts and BL60 transfectants expressing human recombinant APO-1/Fas protein were lysed by anti-APO-1/Fas in a dose-dependent manner. APO-1/Fas is a novel member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfam-

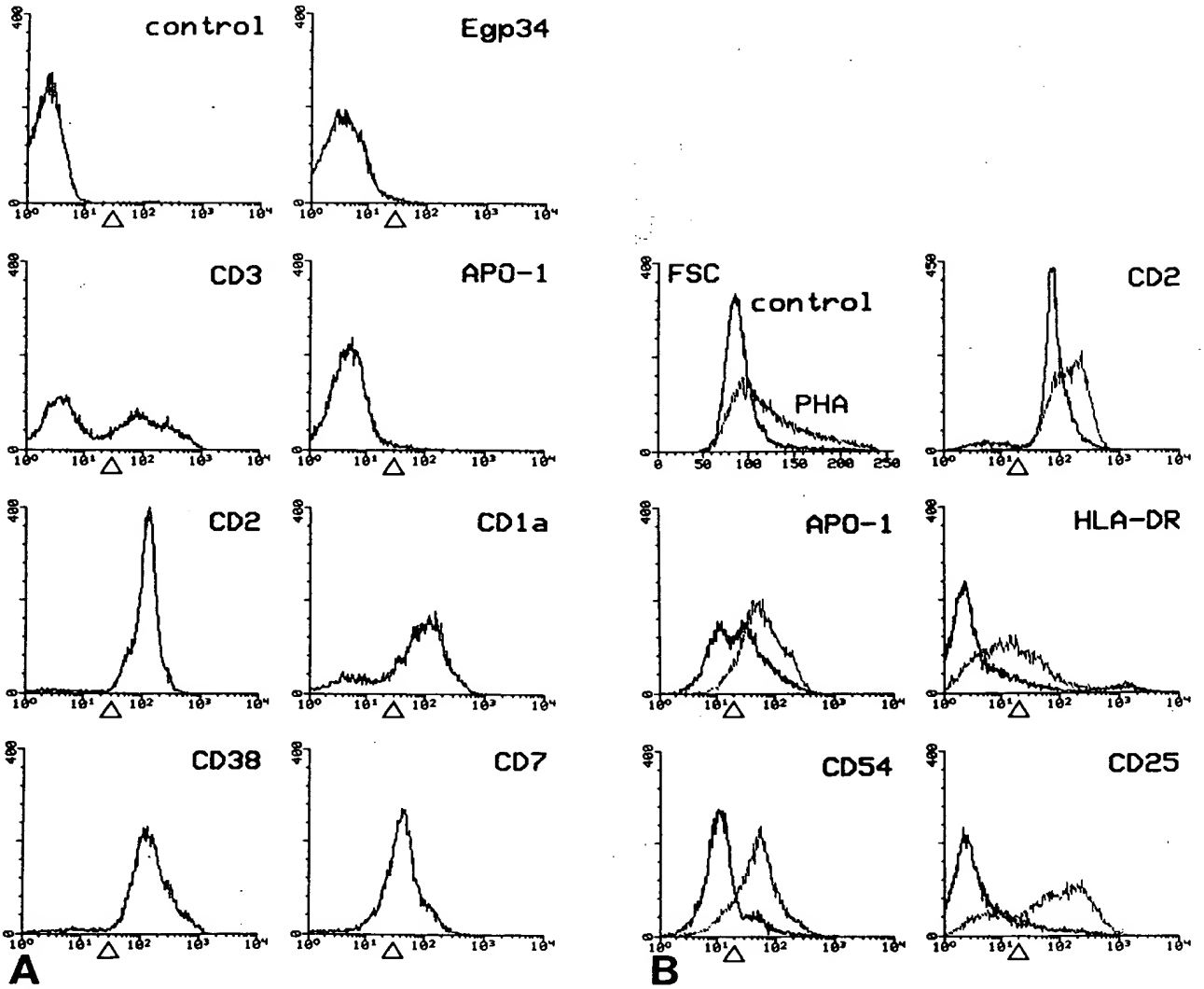

FIG. 6. APO-1 expression on purified thymocytes and peripheral T lymphocytes determined by flow cytometry. *A*, Thymocytes. Thymocytes, showing a typical antigen profile with positivity for CD1a, CD2, CD7 and CD38 as well as heterogeneous CD3 expression corresponding to a mature and an immature thymocyte subpopulation, respectively. These cells do not show APO-1 expression. (Y-axis; relative cell number; X-axis: relative fluorescence intensity; Δ: threshold for positivity defined by a negative control antibody). *B*, Peripheral T lymphocytes unstimulated and stimulated with phytohemagglutinin (PHA-M). (Y-axis: relative cell number, X-axis: cell size (control) or relative fluorescence intensity (antibodies), respectively; Δ: threshold for positivity defined by a negative control antibody). Tonsillar T lymphocytes were stimulated with PHA-M (*hatched line*) (c.f. "*Methods*"). Corresponding cultures kept in medium without PHA-M were used as controls (solid lines). T lymphocytes are identified by strong CD2-expression. Upon PHA-M-stimulation, HLA-DR, CD25 (interleukin-2 receptor), and CD54 (intercellular adhesion molecule 1, ICAM-1) are induced. The admixture of resting and *in vivo* activated peripheral T lymphocytes is APO-1-negative or weakly -positive, respectively. PHA-M-stimulated T cells show an increase in size (forward scatter (*FSC*) histogram) and an elevated expression of APO-1.

ily (6, 7). In order to determine the physiologic function of a receptor, it is essential to know its natural ligand(s). Ligands that under physiologic conditions bind to members of the NGF/TNF-family, have been shown to induce both apoptosis and cell proliferation (16) depending on the kind and functional state of the target cell (17–19). However, for APO-1, the physiologic ligand is yet unknown. Therefore, the question is unresolved whether anti-APO-1 and anti-Fas act as agonists, imitating a natural apoptosis-inducing mediator. Alternatively and analogous the effect of other antibodies on TNF/NGF receptors, both antibodies may exert an antagonistic effect by competitively inhibiting the binding of a survival signal (20) or a growth factor.

Within the immune system, apoptosis occurs predominantly in the thymus (21) and in the center of lymphoid follicles (22). During T cell ontogeny, a large proportion of T cell precursors is negatively selected and dies by apoptosis in the thymus (23). Fas/APO-1 was invoked to be involved in this process in a recent publication by Watanabe-Fukunaga *et al.* (9). These authors showed murine Fas mRNA expression in murine thymic extracts.

Furthermore, they showed that the *lpr* defect in mice is associated with a lack of Fas expression in the thymus

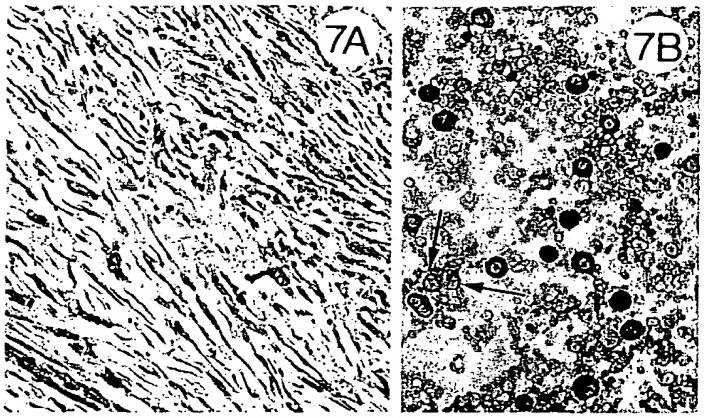

FIG. 7. Expression of APO-1 on mesenchymal cells. *A*, Fresh scar. The connective tissue is composed of APO-1 positive fibroblasts, unstained collagenous fibers and extracellular matrix. *B*, Ascites fluid from a patient with chronic peritonitis. Heterogeneous APO-1 expression of mesothelial cells, the majority showing intense anti-APO-1 staining, whereas few cells are devoid of APO-1 (*arrows*). The smaller, unstained cells mainly represent erythrocytes and lymphocytes. ×129.

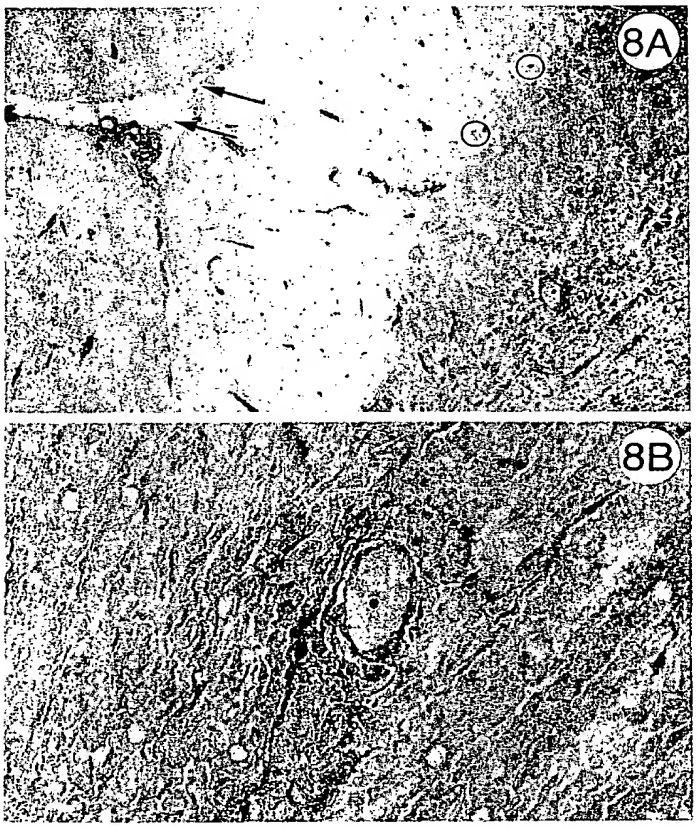

FIG. 8. APO-1 expression in nervous tissue. *A*, Cerebellar cortex. The nerve cell bodies of the stratum granulosum (right) and stratum moleculare (*left*), the Purkinje cells (*circles*) and the glial cells and leptomeninges (*arrows*) are consistently APO-1-negative. Notice the marked APO-1 expression of the vascular endothelium. *B*, Ganglion trigeminale. An APO-1-negative pericaryon is surrounded by a layer of satellite cells exhibiting faint anti-APO-1 staining. Axons and Schwann cells of peripheral nerve truncs are devoid of APO-1. Figure 8*A*, ×64; *B*, ×271.

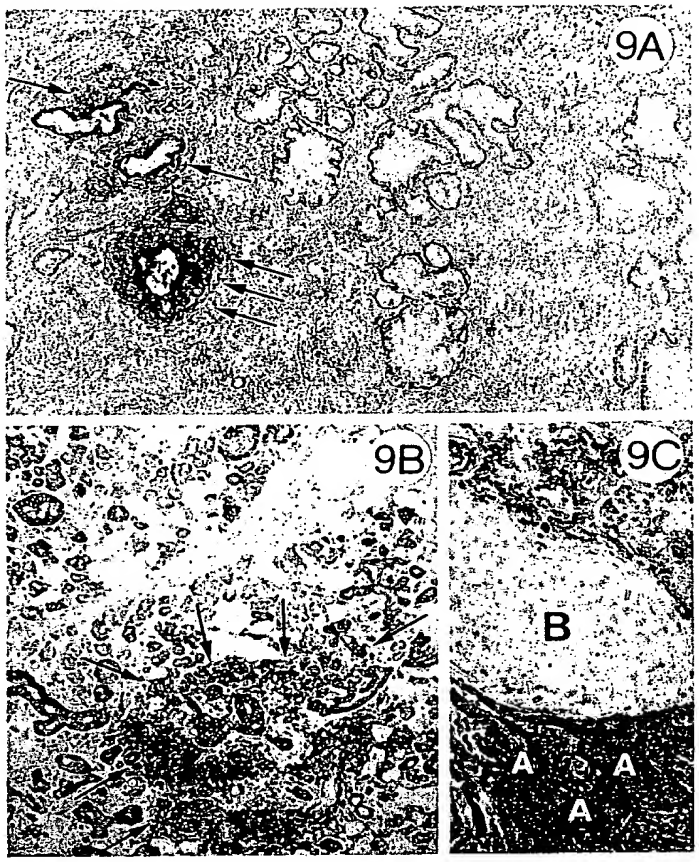

FIG. 9. Induction of APO-1 expression in areas of chronic inflammation. *A*, Chronic prostatitis. Prostatic alveoli situated within dense infiltrates of APO-1 positive lymphocytes show intense APO-1 expression of their epithelial lining as compared with the unstained alveolar epithelium within noninflamed areas. *B*, Chronic sialadenitis. Acinar and ductal cells express APO-1 only in the vicinity of inflammatory infiltrates (*arrows*). The lymphocytes are consistently APO-1-positive. *C*, Chronic orchitis. Lymphocytes of chronically inflamed interstitial tissue (*bottom*) are markedly APO-1 positive while APO-1 is not induced in the adjacent germinal epithelium and Sertoli cells. Figure 9*A* and *B*, ×64; *C*, ×100.

of these mutant mice. The lymphoproliferative and autoimmune syndrome was therefore attributed to defects in negative selection in the thymus. However, our immunohistochemical and flow cytometric data indicate that APO-1 is not expressed on thymocytes *in vivo*. Although APO-1 is induced on activated thymocytes *in vitro* (data not shown), the lack of detectable APO-1 expression on thymocytes argues against an obvious role of APO-1/Fas in thymic selection. The Fas mRNA in thymic extracts described by Watanabe-Fukunaka *et al.* (9) might be derived from thymic epithelial cells rather than from thymocytes themselves. Thus, selection of thymocytes via CD3/T cell antigen receptors (23–25) might involve other molecules than APO-1/Fas.

In B lymphocytes, proliferation and apoptosis are both seen in the follicle center. A high apoptotic rate of B cells was shown to correlate with the absence of *bcl*-2 expression (26, 27). Accordingly, follicle center B blasts are devoid of *bcl*-2 protein (28). Interestingly, APO-1 is expressed in at least a subset of follicle center B blasts.

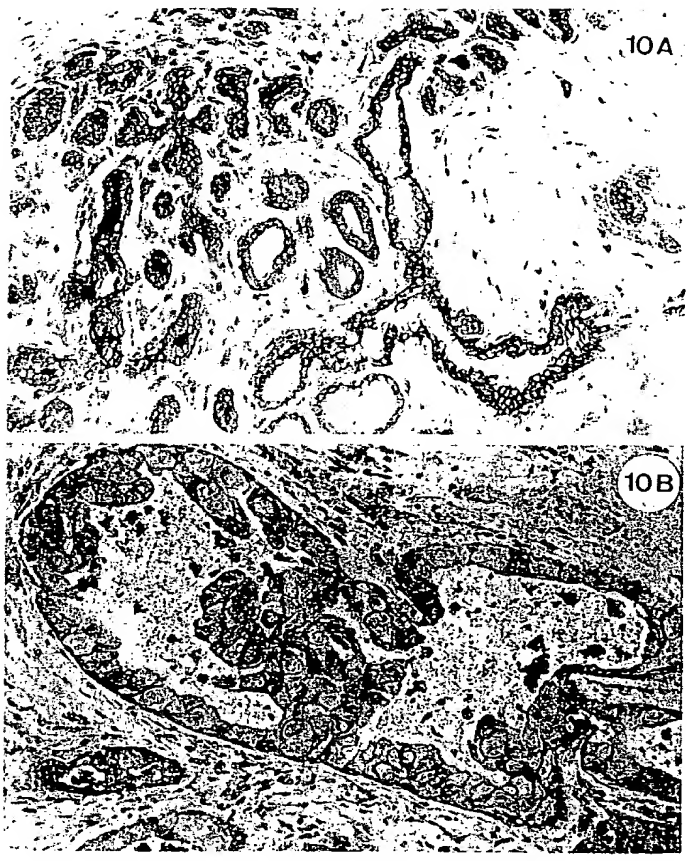

FIG. 10. APO-1 expression in normal and malignant mammary gland. *A*, Normal mammary gland. Heterogeneous anti-APO-1 staining of acinar and ductal epithelium. Most myoepithelial cells of ducts and acini are APO-1-positive. *B*, Ductal carcinoma of the breast. The carcinoma cells show heterogeneous staining and are in part devoid of APO-1 expression. Note again the rim of APO-1 positive myoepithelial cells lining the cancerous ducts. ×129.

This may indicate a role of APO-1 for peripheral B cell selection. Although APO-1 is expressed in B cell areas in which apoptosis occurs, the highest expression of APO-1 was found in sinusoidal B cells. In areas of sinusoidal B cell reaction apoptotic figures are very rare and the proliferation rate is low (P. Möller, unpublished data). Nevertheless, sinusoidal B lymphocytes express the activation-associated molecules CD39 and CD40 at high levels (29). Thus, APO-1 expression might not only be associated with activation-induced apoptosis, but with cellular activation in a more general sense. This interpretation is also supported by APO-1 expression in activated T cells and by the enhanced expression of APO-1 in lymphohistiocytic inflammatory infiltrates. Indeed, as we and others have shown, APO-1/Fas expression is induced by cytokines such as interleukin-2 and IFN-γ alone and in combination with TNF-α (5, 31). Furthermore, APO-1 expression in T cells is enhanced by phytohemagglutinin and in B lymphocytes by cross-linking of surface immunoglobulin in combination with interleukin-2 (30–32). In a number of constitutively APO-1 negative epithelia, we observed APO-1 induction in microtopographical association with chronic inflammatory infiltration. Neoexpression in these sites is likely to be induced by cytokines released by cells of the lymphohistiocytic infiltrate.

In areas of chronic inflammation, parenchymal damage coexists with local proliferation. APO-1 might play a role in both processes. A role in proliferation is supported by expression of this molecule in fibroblasts and mesothelial cells in inflammatory effusions. In fact, in some cell types, the predominant role of APO-1 might be to transduce growth signals. Thus, APO-1 is expressed in proliferating layers of stratified epithelia, but not in superficial layers where cells undergo terminal differentiation and are shedded.

Taken together, our data suggest that in analogy to

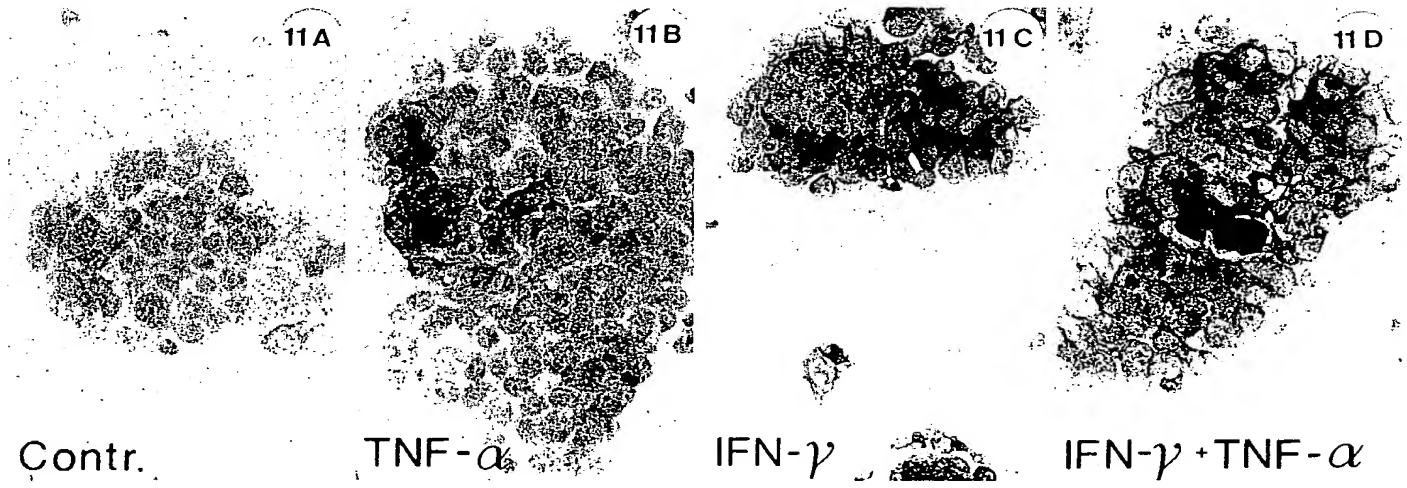

FIG. 11. APO-1 expression of MCF-7 mammary carcinoma cells in the presence of cytokines (IFN-γ and/or TNF-α). *A*, Constitutively, MCF-7 cell line is weakly APO-1-positive in a subset of cells. *B*, APO-1 induction in a subpopulation in the presence of TNF-α. *C*, Intense APO-1 expression in the majority of cells in the presence of IFN-γ. *D*, The combination of TNF-α and IFN-γ has only a slightly enhancing effect on APO-1 expression as compared with IFN-γ alone. ×194.

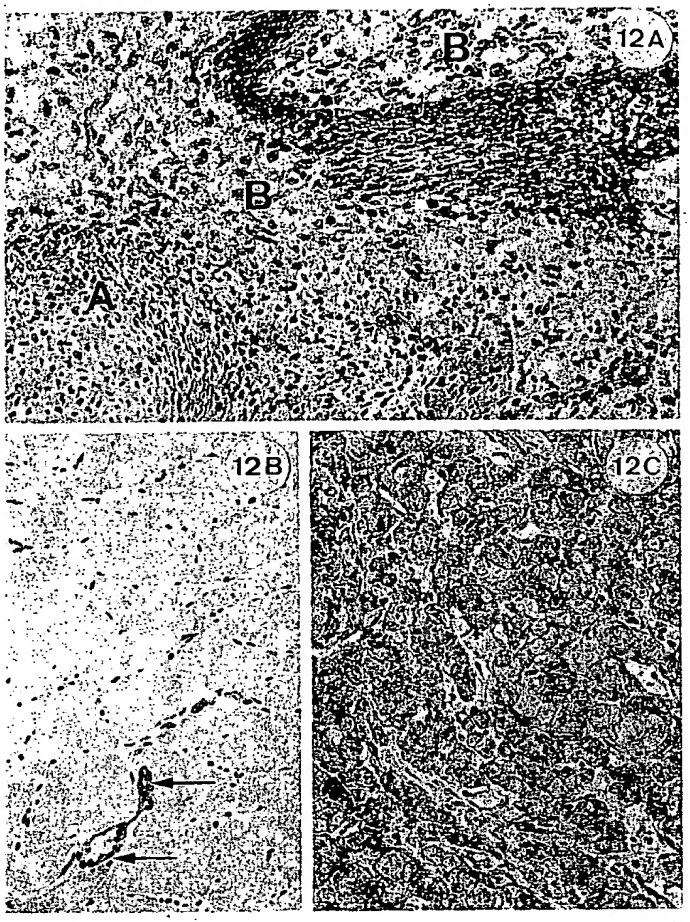

FIG. 12. APO-1 expression in fetal, mature, and malignant skeletal muscle tissue. *A*, Forearm of a fetus. Myoblasts embedded in immature mesenchymal tissue (*B*) show intense APO-1 expression. Notice the onset of cartilage formation (*A*) with strikingly APO-1 positive chrondroblasts. *B*, Histologically mature skeletal muscle of a neonate. No anti-APO-1-staining can be detected on the myocytes, whereas endothelial cells of an interstitial capillary exhibit expression of the APO-1 antigen at an intermediate level. *C*, Rhabdomyosarcoma. Neoplastic cells strongly express APO-1. Figure *A* and *B*, ×129; *C*, ×100.

the TNF receptors APO-1 may serve a dual role and transmit apoptotic and growth signals depending on the target cell type. The issue of the biology of the APO-1 molecule and the physiologic signal(s) transmitted by this molecule will eventually be clarified by the identification of the APO-1 ligand.

METHODS

IMMUNOGLOBULIN CLASS SWITCH

Briefly, the original anti-APO-1 producing hybridoma was established after immunization of BALB/c mice with SKW6.4 B lymphoblastoid cells. The original mAb anti-APO-1 is of the IgG3, κ isotype and is directed against the APO-1 cell surface antigen (1). An IgG1 class switch variant was generated (4) using a method described by Müller and Rajewsky (33).

NORMAL HUMAN TISSUES, TUMOR TISSUES, AND TUMOR TYPING

Normal adult tissues and tumors were drawn from our bank of quick-frozen tissue. Most tissues were obtained from surgical

TABLE 3. EXPRESSION OF APO-1 WITHIN NEOPLASTIC TISSUES AS DETERMINED BY IMMUNOHISTOCHEMISTRY

Type of neoplasm	*N*	+	±	−
Epithelial tumors				
Basal cell carcinoma of skin	1			1
Fibroadenoma of the breast	7			
Ductal cells			4	3
Myoepithelial cells			7	
Carcinoma of the breast				
Ductal (*in situ*/intraductal)	2			2
Ductal invasive	70	51	9	10
Lobular invasive	15	11	3	1
Mucinous	4	3		1
Bronchogenic carcinoma				
Squamous cell carcinoma	2	1	1	
Small cell carcinoma	8			8
Adenocarcinoma	2			2
Large cell carcinoma	2	1		1
Bronchioloalveolar carcinoma	5	1	3	1
Bronchial carcinoid	1			1
Esophageal carcinoma (squamous cell)	2			2
Gastric carcinoma				
Adenocarcinoma	4	2		2
Scirrhous carcinoma	1	1		
Colon adenoma	20	18	2	
Colorectal carcinoma				
Nonmucinous	147	7	55	85
Mucinous	66	22	31	13
Pancreatic carcinoma	2		1	1
Carcinoma of the papilla Vateri	1		1	
Renal cell carcinoma	6	6		
Urothelial carcinoma	1		1	
Endometrial carcinoma	6	4	2	
Miscellaneous tumors				
Nevocellular nevus	4		1	3
Malignant melanoma	11	1	1	9
Malignant mesothelioma				
Fibrous type	1	1		
Epithelial type	1	1		
Mesenchymal tumors				
Leiomyoma	4	2	1	1
Leiomyosarcoma	15	9	2	4
Rhabdomyosarcoma	11	1	2	8
Fibroma	2	2		
Elastofibroma	1			1
Aggressive fibromatosis	4	4		
Fibrosarcoma	5	3	1	1
Synovial sarcoma	7	1	3	3
Malignant fibrous histiocytoma	9	6	3	
Benign schwannoma	3			3
Malignant schwannoma	16	5	3	8
Primitive peripheral neuroectodermal tumor	4			4
Ganglioneuroma	1			1
Liposarcoma	7	3	2	2
Hemangioma	1			
Endothelial component			1	
Fibrous component			1	
Angiosarcoma	1			1
Clear cell sarcoma of tendons and aponeuroses	1			1
Alveolar soft part sarcoma	1	1		
Epithelioid sarcoma	2	2		
Ewing's sarcoma	2			2

continued

TABLE 3. *Continued*

Lymphocyte neoplasias				
Acute B lymphoblastic leukemia	4			4
Chronic B lymphocytic leukemia	11			11
Hairy cell leukemia	8	6		2
Follicular B cell lymphoma				
Mixed small and large cell	17	13	1	3
Small cell	8	6		2
Large cell	11	7		4
Burkitt's lymphoma	8	1		7
Primary gastrointestinal B cell lymphoma				
Low grade malignancy	11	6	2	3
High grade malignancy	17	10		7
Mediastinal (thymic) B cell lymphoma	25	23		2
Plasmacytoma	9	1		8

+ positive, irrespective of staining intensity; ± heterogenous staining, irrespective of percentage of stained cells; − negative.

specimens and biopsies. For most normal tissues two or three samples were examined. Typing of epithelial tumors was carried out according to the guidelines of the WHO (UICC); soft tissue tumors were typed according to the criteria given by Enzinger and Weiss (34) in combination with immunophenotypic data available in each case (35); typing of non-Hodgkin's lymphomas was done using the updated Kiel classification (36) supported by immunophenotyping using CD antibodies (data not shown). As reference organs for non-neoplastic lymphoid tissue, cervical lymph nodes (including several specimens affected by toxoplasmic lymphadenitis), hyperplastic tonsils, and infantile thymus obtained during cardiotomy were used. Among the B cell neoplasias examined were the following specimens: 4 lymph nodes involved by CD10-positive, CD19-positive (data not shown) acute B lymphoblastic leukemia, 11 lymph nodes affected by chronic B-lymphocytic leukemia, 8 spleens and one lymph node involved by hairy cell leukemia, 43 lymph nodes affected by different types of follicular center cell lymphomas, 8 Burkitt's lymphomas of different primary sites, 25 specimens of primary mediastinal (thymic) B cell lymphoma (37–39) and 9 plasmacytomas.

TISSUE PROCESSING

From the quick-frozen tissues serial frozen sections of about 1 cm^2 in area and 4–6 μm in thickness were air-dried overnight, fixed in acetone for 10 minutes at room temperature, and immunostained immediately or stored at −20° C for 1 to 3 weeks.

IMMUNOHISTOLOGIC STAINING PROCEDURE

The method is described in detail elsewhere (40). Monoclonal antibodies were used in a protein concentration of 10 $\mu g/ml$. A polyclonal biotinylated sheep antibody to mouse Ig (reactive with all mouse isotypes) and a streptavidin-biotinylated peroxidase complex, all obtained from Amersham, High Wycombe, United Kingdom, served as detection system for the primary antibodies. 3-Amino-9-ethylcarbazole (Sigma Chemical Company, St. Louis, Missouri) was used as a substrate for the enzyme; the peroxidase reaction resulted in an intense red precipitate. The sections were faintly counterstained with Harris' hematoxylin.

CONTROLS AND EVALUATION OF ANTIGEN DENSITY IN TISSUE SECTIONS

Negative controls were performed without the primary antibody in each individual case and, in a limited number of cases, by employing an irrelevant monoclonal antibody of mouse IgG1 isotype, directed against a non-human antigen. No staining was observed, except for the reaction of granulocytes whose endogenous peroxidase was not destroyed. Strongly stained dendritic stromal cells, histiocytic cells and/or T-lymphocytes, always present in various amounts, served as intrinsic positive controls. To evaluate the neoplastic population within the tumor tissues examined, an evaluation system was designed as follows: positive staining, irrespective of antigen density, was symbolized as "+". Cells that were devoid of any detectable antigen expression were classified as "−". Heterogeneous staining within a given cell population was scored semiquantitatively: more positive than negative cells ("+>−"); approximately equal quantities of positive and negative cells ("±"); more negative than positive cells ("−>+"). For reactive tissue components, the evaluation scheme was extended and the relative staining intensity (roughly corresponding to the antigen density) was also assessed: The staining intensity of interstitial dendritic cells, taken as internal parameter for maximum reactivity, was regarded as "high antigenic density" and symbolized "+". A definitely weaker staining intensity of the target cell population was characterized as "intermediate or low antigenic density" and symbolized "(+)". For comparative visualization of the entire peripheral T or B cell population, a CD3 or a CD19 immunostaining, respectively, was used.

THYMOCYTE AND T CELL PREPARATIONS

A normal juvenile thymus was obtained from thoracic surgery carried out for diagnostic reasons, a juvenile tonsil was obtained from a routine tonsillectomy. Cell suspensions were obtained by mincing the tissues and pressing them through a stainless steel sieve. Mononuclear cells were isolated by Ficoll-Hypaque® (Pharmacia, Piscataway, New Jersey) gradient centrifugation. The mononuclear cells from the thymus preparation were used unfractionated. This cell population was 97% CD2-positive. Tonsillar T cells were isolated via rosetting with 2-amino-ethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC) and Ficoll-Hypaque gradient separation after depletion of monocytes/macrophages by treatment with 2.5 mM L-leucine methyl ester for 40 minutes at room temperature. The T cell/SRBC pellet was resuspended and SRBC were lysed in ammonium chloride lysing buffer. The resulting cell population was 96% CD2-positive.

CELL CULTURE AND STIMULATION

Thymocytes and tonsillar T cells were maintained in RPMI 1640 medium (Gibco, Grand Island, New York) supplemented with 10% fetal calf serum [FCS] (Gibco), 1 mM glutamine, 100 units/ml penicillin, 100 $\mu g/ml$ of streptomycin and 2.5 $\mu g/ml$ amphotericin B and cultured at 37° C in a humidified 5% CO_2 atmosphere. A fraction of the peripheral T lymphocytes was stimulated in the presence of 10 $\mu g/ml$ of phytohemagglutinin-M (PHA-M) (Boehringer, Mannheim, Federal Republic of Germany) for 72 hours.

MONOCLONAL ANTIBODIES FOR FLOW CYTOMETRIC THYMOCYTE/T CELL STAINING

The following monoclonal antibodies were used: CD1a (OKT6), Ortho, Raritan, New Jersey; CD2 (OKT11), Ortho; CD3 (SK7), Becton Dickinson, San Jose, California; CD7 (OKT16), Ortho; CD25 (3G10), Becton Dickinson; CD38 (OKT10), Ortho; CD54 (MEM-112), Serva, Heidelberg, Germany; anti-HLA-DR (ISCR3), kindly provided by the producing laboratory (42), and epithelium-specific anti-Egp34 (HEA125) generated by one of us (42).

FLOW CYTOMETRY

Immunofluorescent staining was performed by using polystyrene round-bottom tubes (Falcon, San Jose, California). Throughout, dilutions and washings were carried out in RPMI 1640 medium (Gibco) containing 2% heat-inactivated FCS, 0.1% sodium azide and 10 mM HEPES buffer. Approximately 10^6 cells/sample, suspended in 50 μl of medium, were incubated at 4° C with an equal volume of the appropriate dilution of each mAb. After 45 minutes, cells were washed twice in 500 μl of cold medium, and 2 μg of $F(ab')_2$ goat anti-mouse IgG and IgM FITC conjugate (Jackson Immunoresearch, West Grove, Pennsylvania) was added for 45 minutes at 4° C. Cells were washed again twice and resuspended in 300 μl of medium containing 1 μg/ml of propidiumiodide (Sigma). From each sample, the green fluorescence of 10^4 cells was analyzed. Dead cells were removed from analysis by selectively gating on red fluorescence, forward, and side scatter parameters. Flow cytometry was performed on a FACScan® cytometer with the LYSYS II software (Becton Dickinson).

MCF-7 STIMULATION

Cells of the MCF-7 mammary carcinoma line (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated fetal calf serum (Gibco), 2 mM L-Glutamine (Gibco), 1% Na-Pyruvate (Seromed, Berlin, Federal Republic of Germany) and 20 mM Hepes buffer, pH 7.3 at 37° C, 5% CO_2 and 95% humidity. After incubating for 72 hours in the presence of 100 units/ml of recombinant IFN-γ (Boehringer, Mannheim, Germany) and/or 1 ng/ml of recombinant TNF-α or medium alone as a control, the cells were harvested from their culture plates by EDTA-treatment and resuspended in culture medium containing 10% fetal calf serum. Cytospin preparations (750 rpm; 5 minutes) were performed using a Shandon cytospin centrifuge (Shandon/Lipshaw, Pittsburgh, Pennsylvania); the slides were fixed for 10 minutes in acetone, air-dried, and stored until staining at −20° C.

Acknowledgement: The excellent technical assistance of I. Brandt, S. Westenfelder, A Müller, and J. Moyers is gratefully acknowledged.

Date of acceptance: April 22, 1993.

This work was supported by grants of the Tumorzentrum Heidelberg/Mannheim, the Deutsche Krebshilfe (989-91; W50/89/Mö 2 and W 8/92/Ko 1), and the Deutsche Forschungsgemeinschaft (KR 776/3-1).

Address reprint requests to: Peter Möller, M.D., Pathologisches Institut der Universität Heidelberg, Im Neuenheimer Feld 220, D-69120 Heidelberg, Germany.

REFERENCES

1. Trauth BC, Klas C, Peters AMJ, Matzku S, Möller P, Falk W, et al. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 1989;245:301–5.
2. Debatin K-M, Goldman CK, Bamford R, Waldmann TA, Krammer PH. Monoclonal antibody mediated apoptosis in adult T cell leukemia. Lancet 1990;335:497–500.
3. Falk MH, Trauth BC, Debatin K-M, Klas C, Gregory CD, Rickinson AB, et al. Expression of the APO-1 antigen in Burkitt lymphoma cell lines correlates with a shift towards a lymphoblastoid phenotype. Blood 1992;79:3300–6.
4. Dhein J, Daniel P, Trauth B, Oehm A, Möller P, Krammer PH. Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on crosslinking of APO-1 cell surface antigens. J Immunol 1992;149:3166–73.
5. Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 1989;169:1747–56.
6. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S-I, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991;66:233–43.
7. Oehm A, Behrmann I, Falk W, Maier G, Klas C, Li-Weber M, et al. Purification and molecular cloning of the APO-1 antigen, a new member of the TNF/NGF receptor superfamily. Sequence identity with the Fas antigen. J Biol Chem 1992;267:10709–15.
8. Lichter P, Walczak H, Weitz S, Behrmann I, Krammer PH. The human APO-1 antigen maps to 10q23, a region which is syntenic with mouse chronosome 19. Genomics 1992;14:179–80.
9. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature (London) 1992;356:314–7.
10. Krammer PH, Debatin KM. When apoptosis fails. Current Biol 1992;2:383–5
11. Sheibani K, Fritz RM, Winberg CD, Burke JS, Rappaport H. "Monocytoid" cells in reactive follicular hyperplasia with and without multifocal histiocytic reactions: an immunohistochemical study of 21 cases including suspected cases of toxoplasmic lymphadenitis. Amer J Clin Pathol 1984;81:453–8.
12. Sohn CC, Sheibani K, Winberg CD, Rappaport H. Monocytoid B lymphocytes: their relation to the patterns of the acquired immunodeficiency syndrome (AIDS) and AIDS-related lymphadenopathy. Hum Pathol 1985;16:979–85.
13. Ahrends MJ, Morris RG, Wyllie AH. Apoptosis. The role of endonucleases. Am J Pathol 1990;136:593–608.
14. Gerschenson LE, Rotello RJ. Apoptosis: a different type of cell death. FASEB J 1992;6:2450–5.
15. Köhler H-R, Dhein J, Alberti G, Krammer PH. Ultrastructural analysis of apoptosis induced by the monoclonal antibody anti-APO-1 on a lymphoblastoid B cell line. Ultrastruct Pathol 1990;14:513–8.
16. Mallett S, Barclay AN. A new superfamily of cell surface proteins related to the nerve growth factor receptor. Immunol Today 1991;12:220–3.
17. Erikstein BK, Smeland EB, Blomhoff HK, Funderud S, Prydz K, Lessauer W, et al. Independent regulation of 55-kDa and 75-kDa tumor necrosis factor receptors during activation of human peripheral blood B lymphocytes. Eur J Immunol 1991;21:1033–7.
18. Heller RA, Song K, Fan N, Chang DJ: The p70 tumor necrosis factor receptor mediates cytotoxicity. Cell 1992;70:47–56.
19. Brakebusch C, Nophar Y, Kemper O, Engelmann H, Wallach D. Cytoplasmic truncation of the p55 tumour necrosis factor (TNF) receptor abolishes signalling, but not induced shedding of the receptor. EMBO J 1992;11:943–50.
20. Raff M: Social controls on cell survival and cell death. Nature (London) 1992;356:397–400.
21. Boyd RL, Hugo P: Towards an integrated view of thymopoiesis. Immunol Today 1991;12:71–9.
22. Liu Y-J, Johnson GD, Gordon J, MacLennan ICM. Germinal centers in T-cell dependent antibody responses. Immunol Today 1992;13:17–21.
23. Finkel TH, Kubo RT, Cambier JC. T-cell development and transmembrane signaling: changing biological responses through an unchanging receptor. Immunol Today 1991;12:79–85.
24. Smith CA, Williams GT, Kingston R, Lenkinson EJ, Owen JT. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature (London) 1989;337:181–4.
25. Takahashi S, Maecker HT, Levy R. DNA fragmentation and cell death mediated by T cell antigen receptor/CD3 complex on a leukemic T cell line. Eur J Immunol 1989;19:1911–9.
26. Hockenbery D, Nunez G, Miliman C, Schireiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature (London) 1990;348:334–6.
27. Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 1991;88:6961–5.
28. Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY. Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 1990;137:225–32.
29. Möller P, Eichelmann A, Moldenhauer G. Surface molecules in-

volved in B lymphocyte function. Virchows Arch A Pathol Anat 1991;419:365–72.
30. Owen-Schaub LB, Yonehara S, Crump WL III, Grimm EA. DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. Cell Immunol 1992;140:197–205.
31. Möller P, Henne C, Leithäuser F, Eichelmann A, Schmidt A, Brüderline S, et al. Co-regulation of the APO-1 antigen with ICAM-1 (CD54) in tonsillar B cells and coordinate expression in subsets of follicular B blasts and follicular center cell lymphomas, and in mediastinal B cell lymphoma. Blood, 1993:2067–5.
32. Mapara MY, Bargou R, Zugck C, Döhner H, Jonker RR, Krammer PH, et al. APO-1 mediated apoptosis or proliferation in human leukemic B cells: correlation with bcl-2 oncogene expression. Eur J Immunol 1993;23:702–8.
33. Müller CE, Rajewsky D. Isolation of immunoglobulin class switch variants from hybridoma lines secreting anti-idiotype antibodies by sequential sublining. J Immunol 1983;131:877–881.
34. Enzinger FM, Weiss SW. Soft tissue tumors. St. Louis: C. V. Mosby Co, 1988.
35. Mechtersheimer G. Towards the phenotyping of soft tissue tumours by cell surface molecules. Virchows Arch A Pathol Anat 1991;419:7–28.
36. Stansfeld AG, Diebold J, Kapauci V, Kelényi G, Lennert K, Miod-uszewska O, Noel H, Rilke F, Sundstrom C, van Unnik JAM, Wright DH. Updated Kiel classification for lymphomas. Lancet 1988;ii:292, 372, 603.
37. Möller P, Lämmler B, Eberlein-Gonska M, Feichter GE, Hofmann WJ, Schmitteckert H, Otto HF. Primary mediastinal clear cell lymphoma of B-cell type. Virchows Arch Pathol Anat 1986;409:79–92.
38. Möller P, Moldenhauer G, Momburg L, Lämmler B, Eberlein-Gonska M, Kiesel S, Dörken B. Mediastinal lymphoma of clear cell type is a tumor corresponding to terminal steps of B cell differentiation. Blood 1989;69:1087–95.
39. Möller P, Matthaei-Maurer DU, Hofmann WJ, Dörken B, Moldenhauer G. Immunophenotypic similarities of mediastinal clear-cell lymphoma and sinusoidal (monocytoid) B cells. Int J Cancer 1989;43:10–16.
40. Mielke B, Möller P. Histomorphologic and immunophenotypic spectrum of primary gastro-intestinal B-cell lymphomas. Int J Cancer 1991;47:334–43.
41. Watanabe M, Suzuki T, Taniguchi M, Shinohara N. Monoclonal anti-Ia murine alloantibodies crossreactive with the Ia-homologues of other mammalian species including humans. Transplantation 1983;36:712–18.
42. Momburg F, Moldenhauer G, Hämmerling GJ, Möller P. Immunohistochemical study of the expression of a M, 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res 1986;47:2883–91.